Supporting Information

Betancur et al. 10.1073/pnas.0906596107

SI Materials and Methods

***Ex Ovo* and *In Ovo* Electroporations.** Chicken embryos were electroporated at stages HH4 to -8 to target the cranial neural crest cell population and at stages HH10 to -12 to target vagal and trunk neural crest (NC) cells following previously described electroporation procedures (1). In *ex ovo* experiments, the DNA plasmid constructs (enhancer-driven reporter with ubiquitously expressing tracer) were introduced in the entire epiblast of the early chicken embryo, while in *in ovo* electroporations only one-half of the neural tube received the DNA. Injected DNA plasmid concentrations were as follows: 2 μg/μL of ptk-EGFP or ptk-Cherry reporter construct, containing each of Sox10 putative *cis*-regulatory regions or the Sox10E2 mutated versions, combined with 1 μg/μL of either tracer (pCI H2B-RFP) or expression constructs (Sox9-pCI H2B-RFP or Ets1-pCI H2B-RFP).

Morpholino-mediated knock-down experiments were performed by injecting the translation-blocking, FITC-labeled morpholino antisense oligonucleotides in one-half of the epiblast (right to the primitive streak) or, in some cases, by double electroporations to differentially transfect each half of the embryo *ex ovo*. Double electroporations were performed by introducing each morpholino combined with the Sox10E2-Cherry plasmid on the right side, and the Sox10E2-Cherry reporter only on the left side of the embryo. Morpholinos used in this study were obtained from Gene Tools and their sequences are as follows: Ets1_5′-GCTTCAGGTCCACCGCCGCCTTCAT-3′; cMyb_5′-ATGGCCGCGAGCTCCGCGTGCAGAT-3′; Sox9_5′- GGGTCTAGGAGATTCATGCGAGAAA-3′; Control_5′-ATGGCCTCGGAGCTGGAGAGCCTCA-3′. The final molar concentration of each morpholino oligonucleotide used was 725 nM.

Microscopy and Inmunohistochemistry. The electroporated embryos were collected at different stages, fixed in 4% paraformaldehyde overnight, and then washed three times in PBS at room temperature. A Zeiss axioskop2 Plus fluorescence microscope equipped with the AxioVision software was employed to image the embryos. Images were processed using Adobe Photoshop CS2. After imaging, embryos were cryo-protected in two steps: 15% sucrose/PBS and 7.5% gelatin/15% sucrose/PBS, equilibrated and mounted in 20% gelatin/PBS and frozen in liquid nitrogen. Next, 12-μm cryosections were collected on Super Frost Plus slides (Fischer Scientific) and degelatinized for 2 × 10 min at 42 °C in PBS. To intensify EGFP signal, the sections were washed four times in PBS for 5 min, blocked for 1 h in 10% Donkey serum/PBTW (PBS/0.1% Tween-20), and stained with 1:1,000 anti-GFP primary antibody (Abcam Inc.) followed by 1:2,000 Donkey anti-goat Alexa-Fluor 488-conjugated secondary antibody (Molecular probes). Sections were subsequently washed, coverslipped, and imaged using the same imaging procedure described for the whole mounts.

Isolation of Regulatory Regions and Cloning. Putative Sox10 regulatory regions were amplified with Expand High Fidelity Plus PCR System (Roche Applied Science), using BAC DNA clones as the template (Chicken BAC library Chori 26, BACPAC Resources). Each fragment, ranging from ~3 kb to 5 kb in size, was cloned into the SmaI-linearized ptk-EGFP vector. The ptk-EGFP reporter vector has the Herpes simplex virus thymidine kinase basic promoter promoter upstream of enhanced GFP and was a kind gift of H. Kondoh (2). The clones with the appropriate orientation were identified by colony PCR and sequenced. The plasmid DNA of the correct clones was prepared and purified using the Endo-free maxi kit (Qiagen) and eluted in EDTA-free buffer.

ptk-Cherry and pCI H2B-RFP plasmids were generated for use in this study. ptk-Cherry reporter vector was made by swapping EGFP with Cherry fluorescent protein in the ptk-EGFP reporter vector (2). pCI H2B-RFP, a tracer construct that yields ubiquitous expression under the control of chicken beta-actin promoter, is a bicistronic vector allowing for exogenous expression of proteins of interest and of a fusion protein of human histone 2B and monomeric RFP protein, translated from the IRES. The pCI H2B-RFP construct was made by replacing the 3xNLS-EGFP sequence within the pCI-GFP vector (3) with the H2B-mRFP1 sequence.

Dissection of Sox10 Downstream Putative Regulatory Region and Mutation of Candidate Binding Sites. The 3.5-kb genomic region downstream *Sox10* coding (Sox10E) was divided into smaller regions, dissected and mutated fragments amplified using Expand High Fidelity Plus PCR System (Roche Applied Science). For the initial dissection of the Sox10E fragment, the following primers were used:

Sox10E1_ 5′, 5′-ATTAGGTACCTCTGATACAGATGCAAGGCTG-3′ and Sox10E1_ 3′, 5′-TAATCTCGAGAATTTGCAGCACTGTGGCCTT-3′; Sox10E2_5′, 5′-AATTGGTACCGGCAAGAGTGGCAATTTAACC-3′ and Sox10E2_3′, 5′-ATTACTCGAGATTGCTTCCCCCTAGACAGTT-3′; Sox10E3_5′, 5′-TTTTGGTACCTAACCAGGGAGGAGTTGTGG-3′ and Sox10E3_3′, 5′-AATTCTCGAGAAGGCCCACAGCAGAGTG-3′. To perturb candidate binding sites within the Sox10E2 fragment, we used single or fusion PCR (4, 5). Only the primers baring the mutations are listed here. Mutated regions are underlined, the mutated nucleotides are shown in bold and fusion primer sequences are italicized:

First two Pax sites clusters (M1, M3)_5′, 5′-AATTGGTACCGGCAA**AGCCCATG**ATTTAACCTACAACTGCTGAGCTTGTAGGA **AGCCCATG**GGCGACTGTGCTTCCGGCT-3′; Myb (M2)_5′, 5′-ATTAGGTACCTGGCAAGAGTGGCAA**GGGATGGACTGGTAGATGGAAG**TGTAGGACTGTGACTGGCGA-3′; Second two Pax cluster sites (M5, M7)_3′, 5′-TCCCTGCTCCTGCTGCTTATCA**TGGGCT**GGGATCCCCTTTCA**TGGGCT**CTGCCCCAGCCGGAAGCACAGT-3′; Ets/Elk (M4)_5′, 5′-ATTAGGTACCTGGCAAGAGTGGCAATTTAACCTACAACTGCTGAGCTTGTAGGACTGTGACTGGCGACTGT**ATGGTTAAT**TGGGGCAGTGCCACTGAAA-3′; NFKB1 (M6)_3′, 5′-TGCTGCTTATCAGTGATG**AGCCCATGGTCT**CAGTGGCACTGCCCCAG3′; Lef/Tcf/SoxE (M8)_3′, 5′-TCTCATCAAATCACCT**CCATCTA**CCCTGCTCCTGCTGCTTATCAGT-3′; Ets (M9)_3′, 5′-AATTCTCGAGATTGCTTCCCCCTAGACAGTTGGGCCTTTGTGCCCTGAGCAGGTTGCTGTGGAAACCCCCAAT**GGG**CTCTCTGGCCAGAGCTGGCT-3′; NFKB1/Lef/Tcf/Ets1_3′, 5′-AATTCTCGAGTTGCTTCCCCCTAGACAGTTGGGCCTTTGTGCCCTGAGCAGGTTGCTGTGG**AGCCCATGGTCT**TCCTCTCTGGCCAGAGC-3′; SoxE/Lef/Tcf (M10)_3′, 5′-ATTACTCGAGATTGCTTCCCCCTAGACAGTTGGGC**G**T**A**TG**C**GCCCTGAGCAGGTTGCTGTGGAAA-3′; Myb (M11)_3′, 5′-ATTACTCGAGATTGCTTCCCCCTA**CTCCATAA**GGCCTTTGTGCCCTGAGCA-3′; SoxD (M12)_3′, 5′-ATTACTCGAG**GCCAA**TTCCCCCTAGACAGTTGGGC-3′.; Δ1_3′, 5′-*AATTTCCTCTCTGGCCAGA* AAATCACCTATTGTTTCCCT-3′; R1_3′, 5′-AATTCCTCTCTGGCCAG**CCTCGGGGTACA**TC**CG**CT**CGGAGG**-**AGGCCTCCCAGCCCATGGTCT**AAATCACCTATTGTTTCCCT-3′. After PCR amplification, each fragment was purified using PCR product purification kit (Qiagen) and cloned into KpnI / XhoI digested ptk-EGFP or ptk-Cherry reporter vectors.

Overexpression Constructs. ORFs of chick *Sox9, cMyb*, and *Ets1* genes were amplified from full-length cDNA clones or chicken cDNA [Sox9 cDNA clone was a kind gift from Yi-Chuan Cheng, Ets1 clone was isolated from the stage 8–12 somites chicken macroarrayed cDNA library constructed by Laura Gammill (6)] and cloned into XhoI/EcoRV or XhoI/ClaI digested pCI H2B-RFP expression vector. The Sox9 and Ets1 rescue constructs were generated from expression constructs by PCR, using primers carrying mutations within the morpholino target sites that do not alter the amino acid sequence of the recombinant proteins. The following primers were used: Sox9_5′, 5′-AATTCTCGAGGCCACCTGCTCAAGGGC-TACGACTGG-3′ and Sox9_3′, 5′-ATTAGATATCTTTAAGG-CCGGGTGAGCTGC-3′; Ets1_5′, 5′-AATACTCGAGGGCCT-CAACCATGAAGGCGGCGGTGGA-3′ and Ets1_3′, 5′-ATTA-GATATCTCACTCATCAGCATCTGGCTTG-3′; cMyb_5′, 5′-A-TTACTCGAGgccacc ATGGCCCGGAGAC; cMyb_3′, 5′-ATT-AATCGATTCACATCACCAGAGTCC; Sox9mut_5′, 5′-ATTAC-TCGAGgccaccATGAActTgtTgGAtCCCTTCATGAAAATGAC; Ets1mut_5′, 5′5′-ATTACTCGAGTCAACCATGAAaGCtGCcG-TcGAttTaAAaCCCACCCTGACCATCA.

Comparative Genomic Analyses. To identify highly conserved genomic regions, the ECR Browser software (http://ecrbrowser.dcode.org) was employed. Chicken, zebrafish, *Xenopus*, opossum, mouse, chimpanzee, dog, rat, and human genomic sequences were downloaded using University of California Santa Cruz genome browser (http://genome.ucsc.edu). Following instructions available on the ECR Web site, these sequences were computationally compared from between all of the species, with conservation parameters set to 70 to 80%. The "zoom in" feature, built in the program, was used to closely analyze the sequence conservation by increasing the threshold up to 90% and using different window sizes ranging from 20 bp to 50 bp.

To search for putative binding motifs, we used the jaspar_core database from Jaspar (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl) and the P-Match program available through Transfac database (http://www.gene-regulation.com/pub/programs.html). Briefly, the 264-bp long sequence of Sox10E2 genomic fragment was uploaded into these programs using desired parameters and the programs returned potential binding motifs based on the position weight matrix (PWM) score. Simultaneously, using rVista 2.0 (http://rvista.dcode.org), Sox10E2 sequence was aligned and compared to the corresponding mouse sequence to screen the latter sequence for conserved putative binding motifs identified in chicken by either Jaspar, Transfac, or both search engines. We used the PWM score, a value given to a site based on the distribution frequency of each base at each position (7), to determine the probability of binding score. This was then used to guide mutational analysis because, as with any informatics approach, there are several caveats: (*i*) this only predicts motifs for factors with known consensus sites; (*ii*) not all functional sites have high PWM scores because they can differ greatly from consensus (8), and (*iii*) not all sites with high PWM are functional.

In Situ Hybridization. Whole-mount in situ hybridizations were performed using a procedure previously described (9). Fluorescent in situ procedure using a GFP probe was adapted from ref. 10. With the exception of the Sox9 and Sox10 probes, which were prepared using full-length cDNA constructs (a gift from Yi-Chuan Cheng) as a template; all other digoxigenin-labeled antisense RNA probes were prepared from chicken EST clones obtained from ARK Genomics and MRC Geneservice. The Sox10 template was digested with HindIII, and all EST clones were linearized using the NotI restriction enzyme. All antisense RNA probes were synthesized using T3 RNA polymerase, according to standard protocols.

ChIP with Sox9, Ets1, and cMyb Antibodies. In ChIP, chromatin precipitation was performed using Ets1 (sc-350; Santa Cruz), Sox9 (ab71762, Abcam, and rabbit polyclonal, from M. Wegner) and cMyb antibodies (11) with normal rabbit IgGs (sc-2027, Santa Cruz; ab27478, Abcam) as negative controls. The protocol was adapted from refs. 12, 13, and http://openwetware.org/wiki/ChIP. For each preparation of nuclei, cranial regions from 20 stage-8 to -12 somite embryos were dissected in Ringer's solution and transferred to 1ml isotonic buffer [0.5% Triton X-100, 10 mM Tris-HCl, pH 7.5, 3 mM CaCl2, 0.25 M sucrose, protease inhibitor tablet (Complete Protease Inhibitor EDTA-free, Roche), 1 mM DTT, and 0.2 mM PMSF] on ice (adapted from ref. 12). Tissue was homogenized using Dounce homogenizer and cells cross-linked by adding formaldehyde to a final concentration of 1% and nutated for 10 min at room temperature. Glycine (final concentration of 125 mM) was added to stop the cross-linking reaction and solution was incubated by nutation for 5 min at room temperature. The cross-linked cells were washed three times and cell pellets were either snap frozen in liquid nitrogen or kept at −80 °C until the ChIP procedure. Preparations were kept up to a month without altering the quality of results. The pellets were resuspended in isotonic buffer and nuclei isolated using Dounce homogenizer, washed, and lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) for 10 minutes to 1 h. The lysate was then diluted 3-fold with ChIP dilution buffer (0.01% SDS, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1 mM DTT, 0.4 mM PMSF, and protease inhibitors) and one-half of chromatin (420 μL) was sonicated using Misonix 4000 sonicator at the following settings: Amp 3, 10 consecutive cycles of 30-s sonication each with 1 min pause in between. Triton X was added to the sonicated material to a final concentration of ∼1%, chromatin was cleared by centrifugation, diluted three to four times with ChIP Dilution Buffer with 1.1% Triton X-100 and was distributed between two to three antibody/bead complexes (400-μL each) and incubated overnight at 4 °C. Fifty microliters of the chromatin preparation was conserved at −80 °C as the input fraction. Antibody/magnetic bead were prepared as per Young protocol (http://openwetware.org/wiki/ChIP). Postimmunoprecipitation washes were performed using RIPA wash buffer (50 mM Hepes-KOH, pH 7.6, 500 mM LiCl, 1mM EDTA, 1% Nonidet P-40, 0.7% Na-Deoxycholate). The complexes were then washed with Tris-EDTA/NaCl (10 mM Tris-HCl, 1 mM EDTA pH 8.0, 50 mM NaCl) for 5 min and transferred to a new chilled tube before last separation (13). The chromatin was eluted in elution buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) and cross-link reversed overnight by incubation at 65 °C. The samples were consecutively treated with RNase A (0.2mg/mL) and then Proteinase K (0.2 mg/mL), extracted with phenol/chloroform/isoamyl alcohol, precipitated, and resuspended in 50 μL of 10 mM Tris pH 8.0. Real-time PCR reactions were performed in a 96-well plate ABI7000 qPCR machine. Reactions were set up using SybrGreen (Biorad), 450 μM of each (forward and reverse primer) and 1 μL of each ChIP reaction or 1:100 to 200 dilution of input fraction. The $\Delta\Delta C_t$ method was used for quantification and calculations performed according to ChIP-qPCR Data Analysis instructions (SupperArray, Bioscience Corporation). To select suitable negative control primers, large regions (potentially corresponding to genomic deserts) of chromosome 1 were surveyed. Because Ets1 binding sites are present in large proportion of the chicken genome, nonspecific binding was a concern; therefore, 8 to 10 different sets of primers were tested. The primers presented in Fig. 6*M* corresponding to the Sox10E2 fragment were: Sox10E2_1 for 5′-TGCTCCTGCTGCTTATCA-3′; Sox10E2_1 rev 5′-ATCAGC TCCACTGCACAT-3′; Sox10E2_2 for 5′-TGATAAGCAG CAGGAGCA-3′; Sox10E2_2 rev 5′-TGAGCAGGTTGCTG TGGAAA-3′. Control primer sets that amplify negative control region situated on the same chromosome as the *Sox10* locus were as follow: negcont_1 for 5′-TCGGATTTTAATGGGCTCAG-3′; negcont_1 rev 5′-CCGCAGATAGTTCTGCATCA-3′ negcont_2 for 5′-GGTTGGATTTCCAGTCTCCA-3′; negcont_2 rev 5′-

TGTTTTGCTGGACAATCTGC-3′. Supplementary technical specifications, primer sequences, and calculation details are available upon request.

EMSA and Pull-Down Assays. EMSA was performed using LightShift Chemiluminescent EMSA Kit (Thermo Scientific) following themanufacturer's instructions. Five Sox10E2 subfragments (S2, S4, S8, S9, S11/S12) and three control fragments were obtained by annealing synthetic oligonucleotides with or without 5′ biotin modification (IDT Biotechnology). Double-stranded fragments used in EMSA assay either had biotin tags on both ends or were not labeled (cold probes).

The sequences of Sox10E2 subfragments used in these approaches were:

S2, GCAATTTAACCTACAACTGCTGAGCTTGTA;
S4, GGCGACTGTGCTTCCGGCTGGGGCAGTG;
S8, GGAGCAGGGAAACAATAGGTGATTT;
S9, TGGCCAGAGAGGAAATTGGGGGTTT;
S11/12, TCAGGGCACAAAGGCCCAACTGTCTAGGGGG.

The sequences of scrambled controls were selected based on the absence of binding sites with homology of greater than 70%, according to exhaustive survey of Jaspar and Transfac databases. They were as follows: Myb Co, TCTTCAAGTCCGCCATGCCCGAAGG; Sox9 Co, TACGGCAAGCTGTTCATCTGCACCA; Ets1 Co, ATGTCTACGTCGAGCGCGACGGCGA.

EMSA. Nuclear extracts from chicken embryos transfected with corresponding expression plasmids (Sox9- Ets1-, cMyb-, or control-pCI H2BRFP) were obtained using standard hypotonic buffer (10 mM hepes pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1× Complete EDTA-Free Protease Inhibitors, 0.2 mM PMSF) to isolate the nuclei and extraction buffer [20 mM hepes pH7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (vol/vol) glycerol, 1mM DTT, 1× Complete EDTA-Free Protease Inhibitors] to obtain nuclear extracts. Binding reactions and gel shift detection was performed using LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

Pull-down assays. Pull-down binding assays were performed using streptavidin Dynal beads (Invitrogen) and the same Sox10E2 subfragments as in EMSA, except that for this experiments the DNA fragments carried a single biotin tag. Dynal beads were coated according to the manufacturer's instructions, using equimolar quantities of the fragment labeled on either the 5′ or 3′ end. Next, 400 to 500 μg of embryonic nuclear proteins (extracted from cephalic regions of chicken embryos at stage HH9–12) in final volume of ∼800 μL of binding buffer [12% glycerol, 12 mM hepes pH 7.9, 4 mM Tris pH 7.9, 150 mM KCl, 1 mM EDTA, 1 mM DTE, and 0.1 μg/μL poly(dI-dC)]. These were pre-exhausted, using ∼1.5 mg of streptavidin Dynal beads and distributed among four Dynal bead preps: two coated with specific DNA fragment, one with scrambled control, and one without DNA. Binding reactions were allowed to proceed for 30 min at room temperature, and were subsequently washed four times with the binding buffer [only the first wash contained poly(dI-dC)]. After the fourth wash, the beads were transferred to a new tube, bound proteins eluted in 30 μL of 50 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA, 1× Protease Inhibitors, 0.1% SDS, and analyzed by Western blot using Sox9, Ets1, and cMyb antibodies.

Cell Death and Proliferation Assays. ***Histology.*** Embryos electroporated unilaterally with single or triple morpholino (at 3-mM concentration) were fixed in 4% paraformaldehyde and dehydrated to 100% methanol. After rehydration, embryos were cryoprotected in 15% sucrose, equilibrated in 15% sucrose/7.5% gelatin, embedded in 20% gelatin, and sectioned at 10 μm using μM cryostat.

TUNEL reaction. The slides were first washed twice in PBS at 42 °C for 10 min to remove gelatin, followed by three to four 10-min washes in PBST (PBS + 0.5% Triton X-100) at room temperature. After 10-min incubation in Permeabilization solution (0.1% sodium Citrate, 0.5% Triton in PBS) and two PBST rinses, the slides were incubated in TUNEL reaction mix (In Situ Cell Death Detection Kit, TMR red, Roche). Labeling solution was first diluted 10 times with TUNEL buffer (30 mM Tris pH 7.2, 140 mM Sodium cacodylate, 1 mM $CoCl_2$) and then combined with enzyme mix as per the manufacturer's instructions (one part enzyme plus nine parts label). A positive control slide, pretreated with DNase I (2 μL of 10 U/μL stock in 100 μL of DNase buffer: 10 mM $CaCl_2$, 40 mM Tris Cl pH7.4, 10 mM $MgCl_2$, 10 mM NaCl, for 1 h at room temperature) was prepared in advance, rinsed with 2 mM EDTA in PBST to quench DNase activity, washed twice in PBST, and stained with TUNEL reaction mix as well. The negative control slide was incubated in TUNEL labeling mix without TdT enzyme. The labeling was performed in the dark for 4 h in a humidified chamber at 37 °C. The slides were then washed 3× PBST for at least 15 min each time; the positive control slide was rinsed in a separate container.

Immunostaining. The slides were incubated in blocking buffer (10% donkey serum in PBST) for 1 h followed by primary antibodies diluted in blocking buffer overnight at 4 °C, [1:1000 rabbit antiPh3 (Phospho-Histone 3) and 1:800 Alexa 488 goat anti FITC]. Slides were then rinsed three to four times in PBST, 30 min each wash. Secondary antibody was diluted in PBST or blocking buffer and applied for 1 h at room temperature. As the TUNEL staining is red (TMR red), we used 1:1000 Alexa 350 goat anti-Rabbit (*blue*) to detect antiPh3, and FITC (morpholino) was labeled in green. All consecutive sections from the cranial region were counted and number of Ph3- (and TUNEL-) positive cells within the neural fold was compared between morpholinoed and control sides for individual, triple, and control morpholinos. We present the mean value of electroporated/control side ratio for triple and control morpholinos. The statistical values were calculated using unpaired Student *t* test.

1. Sauka-Spengler T, Barembaum M (2008) Gain- and loss-of-function approaches in the chick embryo. *Methods Cell Biol* 87:237–256.
2. Uchikawa M, Ishida Y, Takemoto T, Kamachi Y, Kondoh H (2003) Functional analysis of chicken Sox2 enhancers highlights an array of diverse regulatory elements that are conserved in mammals. *Dev Cell* 4:509–519.
3. Megason SG, McMahon AP (2002) A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* 129:2087–2098.
4. Szewczyk E, et al. (2006) Fusion PCR and gene targeting in *Aspergillus nidulans*. *Nat Protoc* 1:3111–3120.
5. Heckman KL, Pease LR (2007) Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat Protoc* 2:924–932.
6. Gammill LS, Bronner-Fraser M (2002) Genomic analysis of neural crest induction. *Development* 129:5731–5741.
7. Hollenhorst PC, Shah AA, Hopkins C, Graves BJ (2007) Genome-wide analyses reveal properties of redundant and specific promoter occupancywithin the ETS gene family. *Genes Dev* 21:1882–1894.
8. Bajard L, et al. (2006) A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. *Genes Dev* 20: 2450–2464.
9. Wilkinson DG (1992) Whole mount in situ hybridisation of vertebrate embryos. *In situ Hybridization: A Practical Approach*, ed Wilkinson DG (IRL Press, Oxford), pp 75–83.
10. Acloque H, Wilkinson DG, Nieto MA (2008) In situ hybridization analysis of chick embryos in whole-mount and tissue sections. *Methods Cell Biol* 87:169–185.
11. Karafiat V (2005) et al.Transcription factor c-Myb is involved in the regulation of the epithelial-mesenchymal transition in the avian neural crest. *CellMol Life Sci*62), 62pp 2516–2525.
12. Buchholz DR, Paul BD, Shi Y-B (2006) *Chromatin Immunoprecipitation for in vivo Studies of Transcriptional Regulation During Development. Analysis of Growth Factor Signaling in Embryos, Methods in Signal Transduction*, eds Whitman M, Sater AK (CRC Press, Florida), pp 257–271.
13. Dahl JA, Collas P (2008) A rapid micro chromatin immunoprecipitation assay (microChIP). *Nat Protoc* 3:1032–1045.

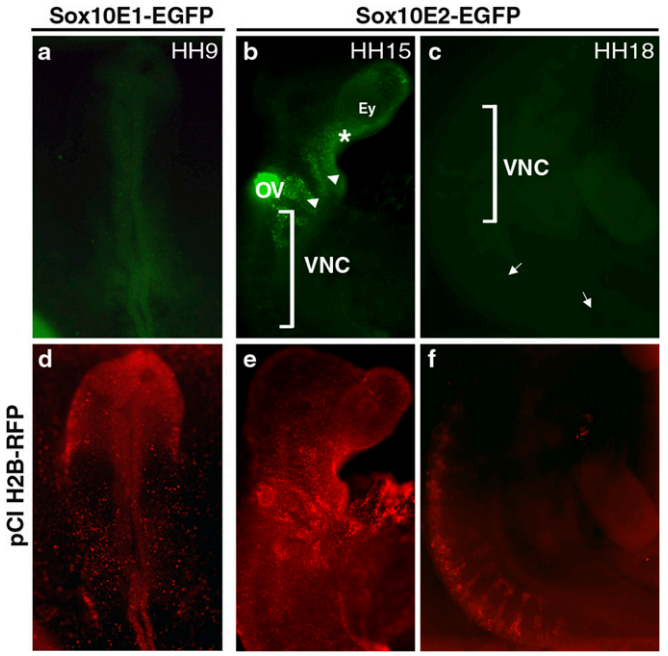

Fig. S1. Dissection of Sox10E reveals two regulatory regions that function in a specific spatiotemporal manner: Sox10E2 is activated as cranial neural crest cells delaminate, whereas Sox10E1 is activated in later migrating vagal and trunk NC. (*A*) Sox10E1 displays no activity in the delaminating cranial neural crest. (*B*) Sox10E2 activity at HH15 persists in the periocular crest and otic vesicle, but also within the first two branchial arches, which lack endogenous Sox10; Sox10E2 is not expressed in vagal NC at this stage or later, at HH18 (*C*) in either the vagal or trunk regions. (*D–F*) Panels corresponding to *A* to *C*, respectively, show expression of the coelectroporated tracer pCI H2B-RFP to locate cells that received both tracer and reporter EGFP plasmid DNA.

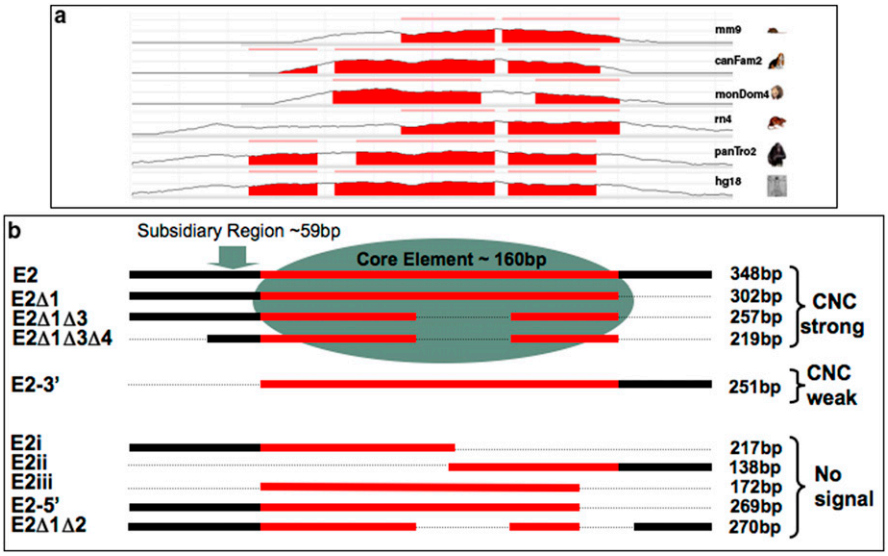

Fig. S2. Dissection of the Sox10E2 fragment reveals an essential core and auxiliary region important for optimal enhancer activity. (*A*) "Zoomed in" image showing the region Sox10E2 from the genomic comparative analysis illustrated in Fig. 2*A*. Each uncolored peak represents the conserved region equivalent to region Sox10E2 in chicken for each corresponding species. Inside the conserved regions, the red patches represent highly conserve portions (85–90%, each 30 bp). The order of species from top to bottom: mouse, dog, opossum, rat, chimpanzee, and human. (*B*) Schematic diagram representing the different successive deletions (*dotted lines*) that were performed, guided by bioinformatics, to identify the main core element responsible for the regulatory activity of the Sox10E2 fragment. The horizontal black lines represent different fragments. The red portion of the lines denote a 160-bp region (referred to as the "essential core element"), highly conserved between dog, chimpanzee, and human, capable of producing weak tissue-specific regulatory activity in delaminating cranial NC cells. The gray arrow points to a nonconserved 59-bp long auxiliary region, necessary for achieving strong regulatory activity of the enhancer. CNC, cranial neural crest.

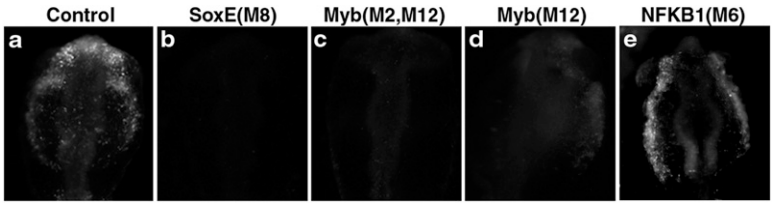

Fig. S3. Transcriptional inputs into the Sox10E2 regulatory region. (*A*) EGFP pattern of expression in neural crest cells driven by the intact Sox10E2 regulatory region (*B* and *C*). EGFP expression (*B*, *green*) is abolished in cranial NC when binding motifs for putative upstream regulators, SoxE (*B*) and a pair of Myb (*C*), are mutated (mutations M8 and M2/M12, respectively). (*D*) A mutation of a single Myb binding site mildly decreases EGFP reporter expression, while a NFkB1 mutation does not affect reporter signal (*E*).

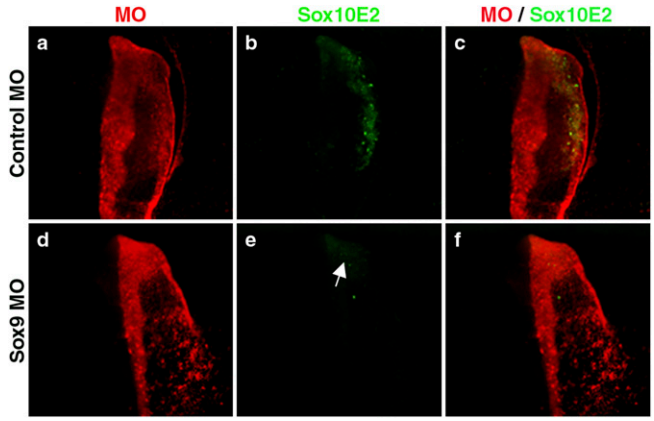

Fig. S4. Morpholino-mediated Sox9 knock-down significantly reduces Sox10E2 regulatory activity. (*A*) FITC-labeled control morpholino (red) does not affect Sox10E2 driven Cherry expression (green) (*B*). (*C*) Merged image of *A* and *B* shows overlap of control morpholino (red) with Sox10E2 driving expression of Cherry (green). (*D*) Sox9 FITC-labeled morpholino (red) strongly reduces Sox10E2 driven Cherry expression (green, white arrow) (*E*). (*F*) Merged image of *D* and *E* shows the effect of Sox9 morpholino (red) on reporter expression of Cherry driven by Sox10E2 regulatory region (green). Embryos were electroporated on the right side only. The images were pseudocolored using Photoshop, with green and red channels inverted for consistency, indicative of reporter expression.

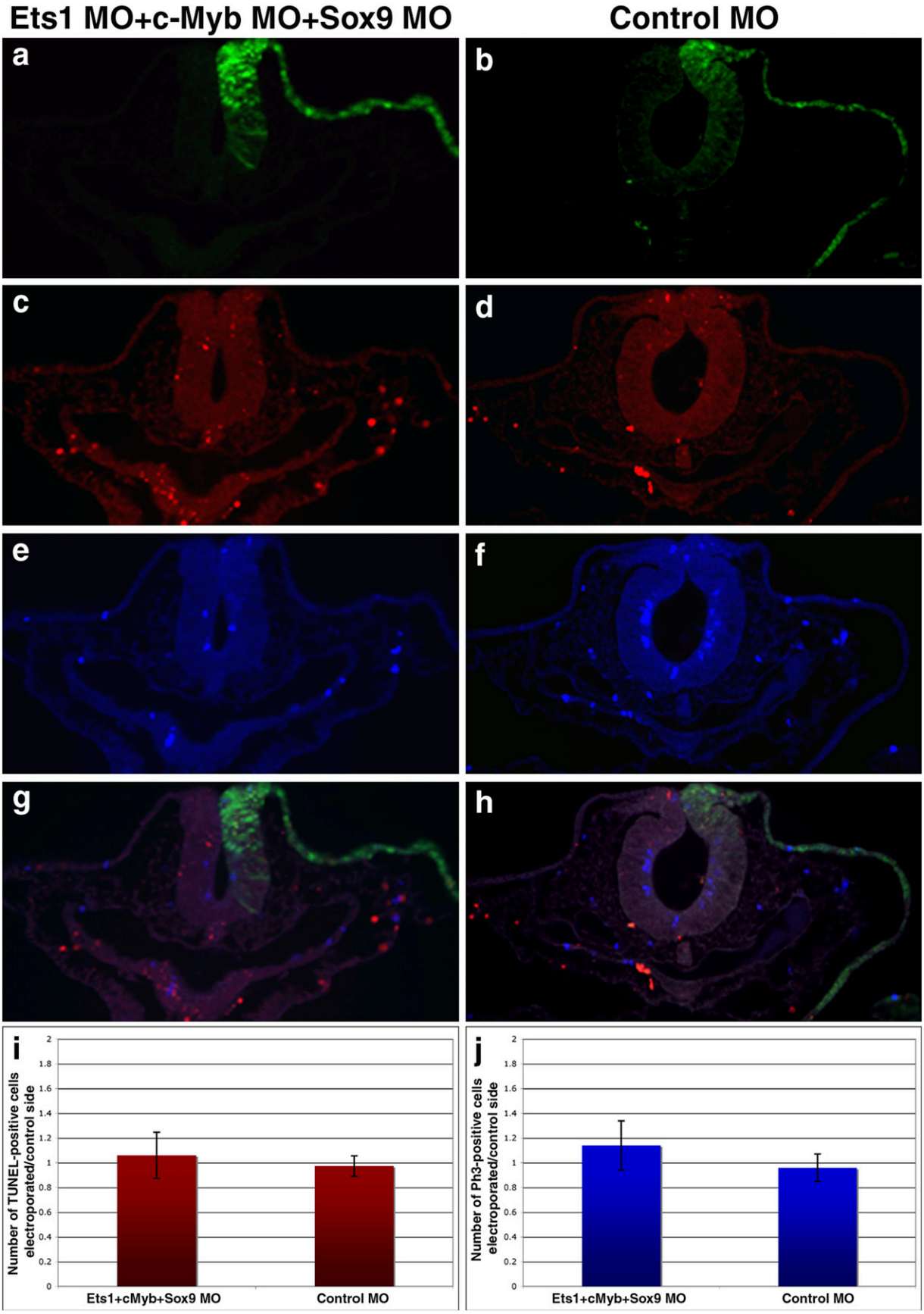
Ets1 MO+c-Myb MO+Sox9 MO
Control MO
a
b
c
d
e
f
g
h
i
Number of TUNEL-positive cells electroporated/control side
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ets1+cMyb+Sox9 MO
Control MO
j
Number of Ph3-positive cells electroporated/control side
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ets1+cMyb+Sox9 MO
Control MO

Fig. S5. Electroporation of Ets1, cMyb, Sox9, and control morpholinos does not affect cell proliferation and does not induce apoptotic cell death. Embryos electroporated with FITC-labeled triple (Ets1, cMyb, and Sox9) or control morpholinos (*A* and *B*) were sectioned and TUNEL staining (*C* and *D*) or anti-Phospho-Histone H3 (Ph3) antibody staining (*E* and *F*) was performed on sections. (*G* and *H*) Overlay of FITC, TUNEL and Ph3 staining presented in (*A*, *C*, and *E*) and (*B*, *D*, and *F*), respectively. All consecutive sections from the cranial region were counted and the number of Ph3- (and TUNEL-) positive cells within the neural fold was compared between morpholinoed and control sides. Mean value and standard deviation of electroporated/control side ratio from four independent embryos are presented [(*I*) TUNEL, *green bars*; (*J*) Ph3, *blue bars*]. The statistical calculations performed using unpaired student's *t*test show no statistically significant differences in cell death or proliferation counts between electroporated and control sides of embryos receiving either three specific morpholinos or control (at 3 mM).

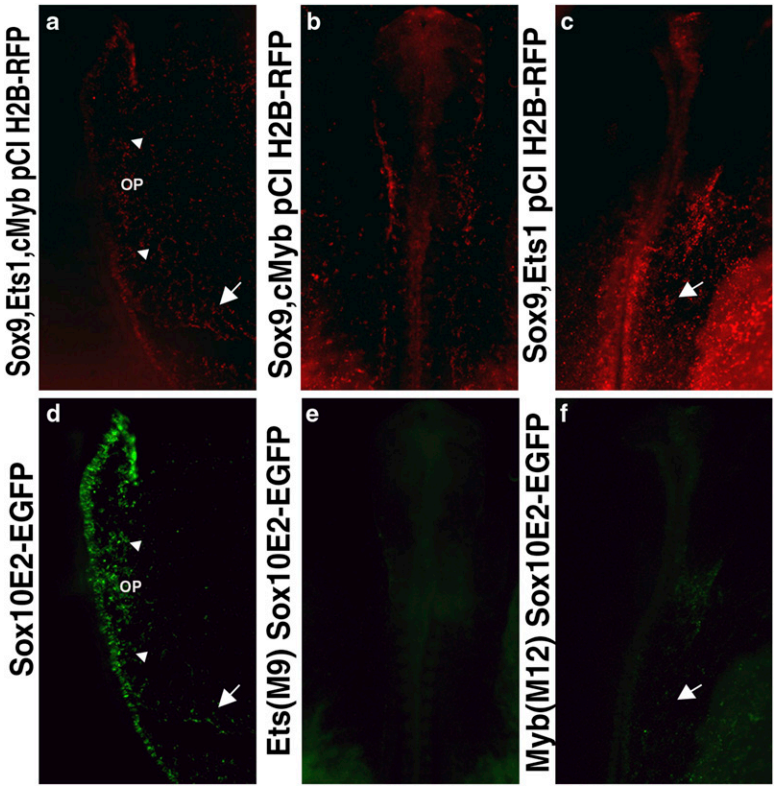

Fig. S6. Binding motifs for SoxE, Ets, and Myb within Sox10E2 enhancer need to be functional for ectopic reporter expression to occur when misexpressing Sox9, cMyb, or Ets1, individually or in combination. (*A* and *D*) Ectopic EGFP expression is observed in the extraembryonic region, ectoderm cells (arrowheads) and along the neural tube (*D*), when Sox9-pCI H2B-RFP, Ets1-pCI H2B-RFP, and cMyb-pCI H2B-RFP (*red*) are simultaneously overexpressed (*A*). Combined Sox9 and cMyb misexpression (*B*) fail to activate ectopic EGFP expression through a mutated Sox10E2 regulatory region (M9) that lacks an Ets binding motif (*E*), whereas overexpressing Sox9 and Ets1 simultaneously can activate weak reporter expression (arrows) through a mutated Sox10E2 lacking one Myb (M12) binding motif.

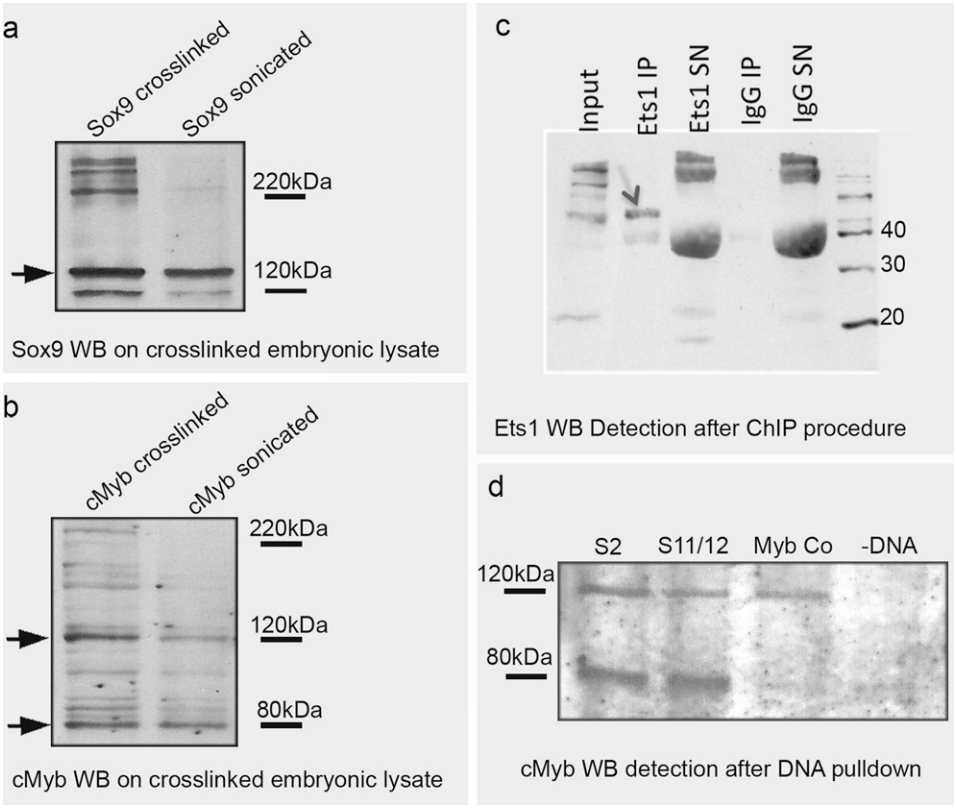

Fig. S7. Ets1, cMyb, and Sox9 antibodies recognize cross-linked endogenous corresponding proteins. (*A* and *B*) Western blots, using Sox9 and cMyb antibodies on cross-linked or cross-linked/sonicated material, performed under moderate denaturing conditions. Specific bands were noted at ~120 kDa for Sox9 and at ~80 kDa and 120 kDa for cMyb. Similar Sox9 and cMyb protein bands were detected after pull-down assays. The 120-kDa cMyb protein was also bound to cMyb scrambled control (Myb CO) with 70% homology threshold (*D*). Partial denaturating conditions show that other less prominent bands likely represent complex associations rather than Sox9 or cMyb bands, as they are less prominent in the sonicated condition. (*C*) ChIP using Ets1 antibody, followed by Western blot using the same target antibody. Detection was performed using IP-Western kit (GenScript) and shows several bands in cross-linked and sonicated input, under moderate denaturation conditions, with a single Ets1-precipitated band (~*arrow*) detected in the ChIP and input lanes, but not in samples precipitated with IgG antibody. IgG IP, immunoprecipitation with normal rabbit IgGs (negative control); IP, Immunoprecipitated fraction; S2, subfragment of Sox10E2 enhancer containing N-terminal Myb site (see *SI Materials and Methods* for sequence details); S11/12, subfragment of Sox10E2 enhancer containing C-terminal Myb site; Myb CO, scrambled control fragment containing no Myb sites with >70% homology; -DNA, control pull down using streptavidin magnetic beads only, without DNA fragment; SN, first supernatant.

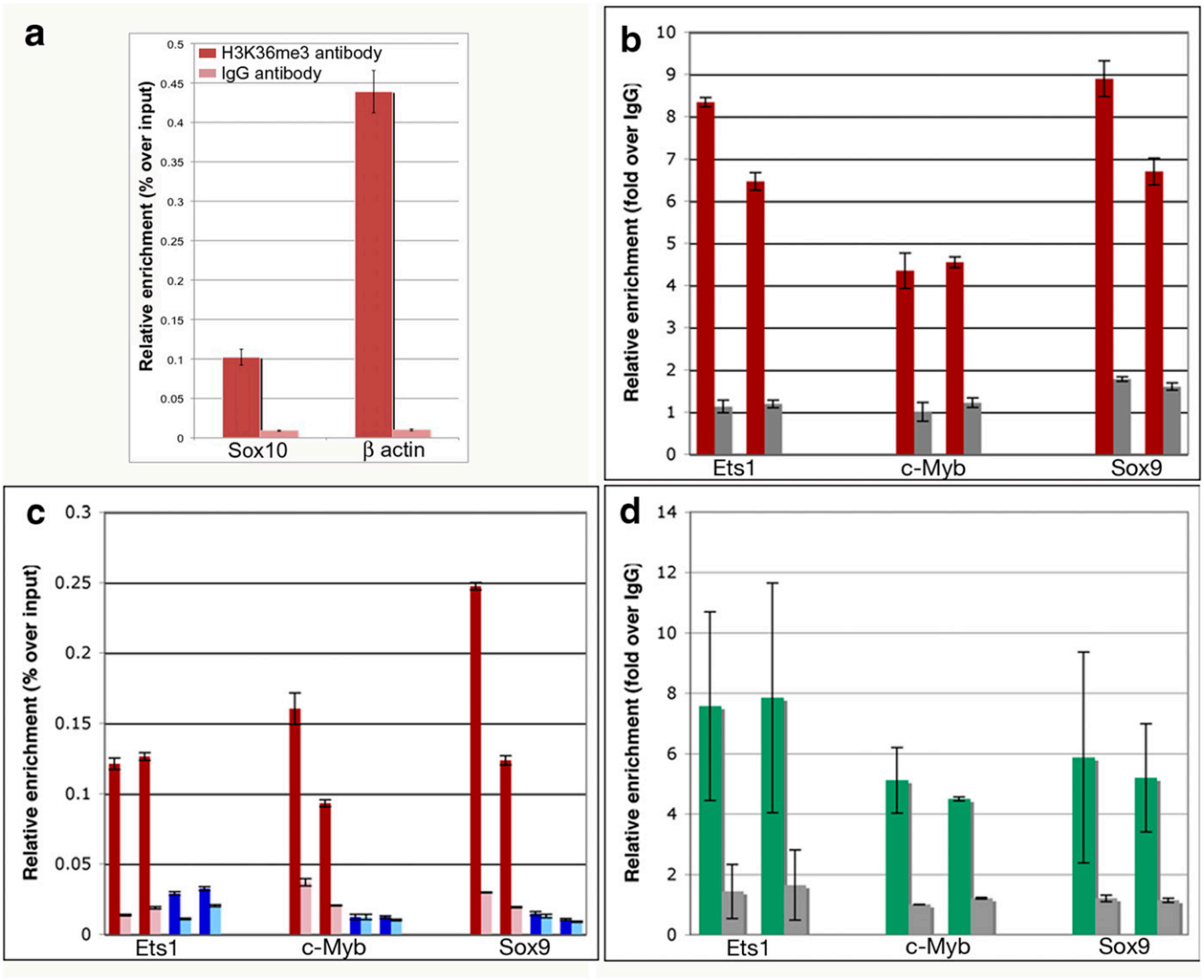

Fig. S8. Ets1, cMyb, and Sox9 bind directly to Sox10E2 enhancer element in vivo. Direct binding of Ets1, cMyb, and Sox9 transcription factors to the Sox10E2 regulatory element driving the onset of Sox10 expression in the delaminating NC was assessed using qChIP. (*A*) qChIP-positive control using antibody against H3K36me3, chromatin mark generally associated with active gene transcription. ChIP shows high occupancy of H3K36me3 mark in proximity of the transcriptional start site of Sox10 and control active β-actin locus. Relative enrichment over input using specific and negative control antibodies (normal rabbit IgGs) are presented. (*B*) qChIP results obtained using specific target antibodies (Sox9, Ets1, and cMyb) to precipitate chromatin regions specifically bound by those factors. The results presented as relative enrichment over input using specific or negative control antibody (normal IgGs) were obtained by quantitative PCR where the same amount of DNA from each pull-down was used in a separate reaction and two primer sets specific with Sox10E2 region (*red bars*) and two primer sets within negative nonbound region (*gray bars*). (*C*) Enrichment is expressed relative to input DNA using the same amount of DNA in the qPCR reaction for each ChIP. Enrichment of the specific factors (Ets1, cMyb, or Sox9) at Sox10E2 enhancer region is presented as red bars; enrichment of specific factor at negative control regions is presented in blue. Background levels (mean enrichment from control antibodies) at enhancer and negative control regions are shown as pale red or pale blue bars, respectively. (*D*) Means and standard deviations of relative enrichment (fold over negative control antibody) are presented from three to four independent experiments for each specific antibody used. Green bars represent enrichment at Sox10E2, gray bars at negative control regions.

Table S1. List of primers used to amplify putative regulatory regions surrounding the *Sox10* locus

Fragment	5′ primer	3′ primer	Genomic position	Predicted size
Sox10_5	GTGTAGAGCCCGGTGGTG	AAGCAACTCACCGCCATC	Chr. 1 52975810–52978750	2,941
Sox10_6	TGCAGAAAGCATGGCAGA	CACCAGGTGCCAACACAA	Chr. 1 52979561–52982340	2,780
Sox10_7	CCAGCTCCCTCAGCCTTT	ATGCCACATCCCTGGAAA	Chr. 1 52985449–52990190	4,742
Sox10L8	**GATGCCTGGATGGTGCTC**	**TTCAGTGCTTTGCCACCA**	**Chr. 1 52990575–52993590**	**3,016**
Sox10_9	GTGCTGGTGAGCCGAACT	GAGGGCAAGCACCTCAGA	Chr. 1 52992960–52994741	1,782
Sox10_10	GCCGTGTGTCTTCCCATC	ATCCCCACCACGGAGTCT	Chr.1 52995526–52999473	3,948
Sox10E	**GGGGATACTGGCCTGCTT**	**AAGGCCCACAGCAGAGTG**	**Chr.1 53010305–53014595**	**4,291**

High-fidelity PCR approach was used to amplify genomic regions containing putative conserved regulatory elements controlling Sox10 expression in the chicken embryo. Two fragments (in bold) showed enhancing activity at later (Sox10L8) and early (Sox10E) steps of neural crest formation.