Supporting Information

Liu et al. 10.1073/pnas.1211747110

SI Materials and Methods

Chicken Embryos. Fertilized chick eggs were obtained from Jinan Poultry Co. (Tin Hang Technology) and were incubated at 38 °C in a humidified incubator (Brinsea Octagon 250 incubator). Embryos were staged according to Hamburger and Hamilton (HH) (1).

Expression Vectors and Morpholinos. Point mutations were introduced into two phosphorylation sites (S64 and S181) or three SUMOylation consensus sites (K61, K254, and K376) of chick *Sox9* full-length cDNA using the PCR-based QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol. These cDNAs were inserted upstream of an internal ribosomal entry site (IRES) and a nuclear localization sequence (nls)-tagged GFP in a pCIG expression vector (kind gift of A. McMahon, Harvard University, Cambridge, MA). Primer sequences for mutagenesis are available on request. The V5-epitope tag was engineered into the N terminus of chick *Snail2* and UBC9 full-length proteins, and both cDNAs were PCR-amplified from oligo(dT)-primed HH10-12 embryos cDNAs. Smad6, CA-BMPR-1A, β-catenin-EnR, and TCF-VP16 were cloned into the pCIG-IRES-nlsGFP vector (kind gift of N. Itasaki, University College Dublin, Dublin). Two tethered constructs were generated by fusing the *Sox9* or $Sox9^{S64A,S181A}$ coding region in frame with *Snail2* cDNA with a glycine-serine linker (GSGGGGSGGGGSG) in between and cloned into the pCIG-IRES-nlsGFP vector.

Sox9 and control morpholinos used in this study were obtained from Gene Tools, and their sequences are as follows: *Sox9*_5′-GGGTCTAGGAGATTCATGCGAGAAA-3′ and control_5′-ATGGCCTCGGAGCTGGAGAGCCTCA-3′. The final molar concentration of each morpholino oligonucleotide used was 725 nM.

The *Sox9* and its phospho-mutant rescue constructs were generated from expression constructs by PCR, using primer carrying mutations within the morpholino target sites that do not alter the amino acid sequence of the recombinant proteins. The following primer was used with site of mutations in lowercase letters: 5′-ATTACTCGAGgccaccATGAActTgtTgGAtCCCTTCATGAAAATGAC.

In Ovo Electroporation and Chick Neural Tube Explant Culture. In ovo electroporation were carried out as described (2). Briefly, plasmid DNA was injected into the lumen of HH stages 11–12 neural tubes, electrodes were placed on either side of the neural tube, and electroporation was carried out using a BTX electroporator delivering five 50-ms pulses of 30 V. Electroporated embryos were incubated for 24 or 36 h posttransfection (hpt) before being processed for Western blotting analysis, immunohistochemistry, and in situ hybridization. For neural tube explant culture, embryos were harvested at 2 hpt, and neural tubes at the level of recently formed three somites were carefully dissected following dispase (Roche) treatment, explanted onto fibronectin-coated eight-well slides before culturing in F12-based medium with L-glutamine (Gibco), 1% N2 supplement (Gibco), and 1% penicillin-streptomycin (Gibco) for 24 h at 37 °C and 5% CO_2. Subsequently, the neural tube explants were fixed for 30 min in 4% paraformaldehyde (PFA), immunostained with HNK-1 and DAPI stained, and photographed using a Zeiss LSM700 confocal microscope from the Faculty Core Facility, Li Ka Shing Faculty of Medicine, University of Hong Kong.

Frozen Sectioning, In Situ Hybridization, and Immunohistochemistry. Electroporated embryos were harvested and fixed for 1 h at 4 °C in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), cryoprotected with 30% sucrose in PB, and cryosectioned at 12 μm. Immunohistochemical localization of proteins on sections and neural plate explants were performed as described (3). Antibodies against the following proteins were used: GFP (Molecular Probes), Sox2 (Neuromics), *Sox9* (gift from V. Lee Medical College of Wisconsin, Milwaukee), HNK-1 (Becton Dickinson), *Sox9*p^{S181} (Abcam), *N*-Cad (Zymed), aPKC (Santa Cruz), *Snail2* (Cell Signaling), Capase3 (Cell Signaling), and Laminin (Sigma). Images were collected as described above.

Whole-mount in situ hybridization was performed as described (2). The following probes were used: chick FoxD3 (I.M.A.G.E. ID 418507), chick *Sox9* 3′UTR, chick Sox10 3′UTR (3), and chick *Snail2* (gift from A. Nieto, Instituto de Neurociencias, San Juan de Alicante, Spain).

Western Analysis and Coimmunoprecipitation. For each *Sox9* mutant variant, 15 well-transfected chicken embryos at stages HH 14–16 were harvested and lysed with lysis buffer, complete protease inhibitor (Roche), and 40 mM *N*-ethylmaleimide to inhibit endogenous deSUMOylase activity. For the detection of SUMOylated *Sox9* protein, embryo crude extracts was subjected to 10-s sonication with 25% amplitude. For coimmunoprecipitation studies, 0.2% sodium deoxycholate was added into lysis buffer without sonication. Western blotting was performed based on standard procedures. Preclearing of chicken embryo lysates was performed by incubating with protein G Sepharose beads (GE Health Care) for 16 h at 4 °C, followed by overnight immunoprecipitation with mouse anti–Flag-M2 (Sigma) or rabbit anti-*Sox9* (Millipore). The protein–antibody complex was then incubated with protein G Sepharose beads for 3 h at 4 °C. The immunoprecipitated protein complex was immunblotted with the following primary antibodies overnight at 4 °C: rabbit anti-*Sox9* (1:2,800; Millipore), rabbit anti-*Sox9*p^{S181} (1:1,000; Abcam), mouse anti–GMP-1 (1:500; Zymed), and rabbit anti-V5 (1:1,000; Invitrogen). Quantification of band intensities in Western blot was performed in Photoshop CS2 to compare the changes of protein amount between samples.

1. Hamburger V, Hamilton HL (1992) A series of normal stages in the development of the chick embryo. 1951. *Dev Dyn* 195(4):231–272.
2. Cheung M, Briscoe J (2003) Neural crest development is regulated by the transcription factor Sox9. *Development* 130(23):5681–5693.
3. Cheung M, et al. (2005) The transcriptional control of trunk neural crest induction, survival, and delamination. *Dev Cell* 8(2):179–192.

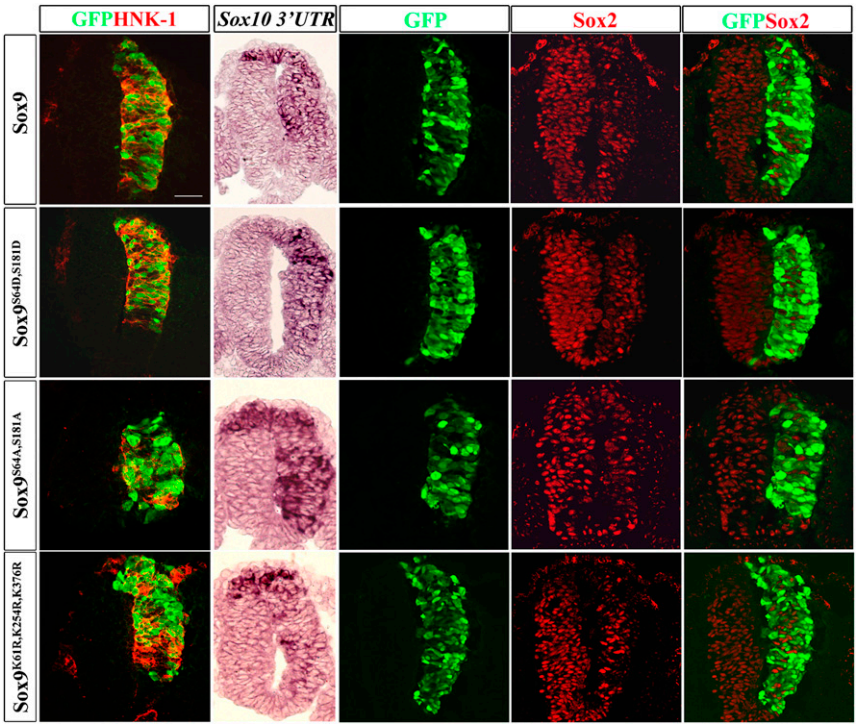

Fig. S1. *Sox9*, regardless of its phosphorylation and/or SUMOylation status, is able to induce the switch from neural progenitor to neural crest (NC) fate before the onset of delamination. Chicken embryos at stages HH 11–12 were electroporated into the caudal hemineural tube with the indicated constructs and analyzed by in situ hybridization with Sox10 3′UTR and immunofluorescence with human natural killer-1 (HNK-1) and Sox2 at 24 hpt. Electroporation of WT-*Sox9*, *Sox9*^{S64D,S181D}, *Sox9*^{S64A,S181A}, or *Sox9*^{K61R,K254R,K376R} causes induction of HNK-1 and Sox10 expression, whereas Sox2 is repressed in a cell-autonomous manner. (Scale bar, 50 μm.)

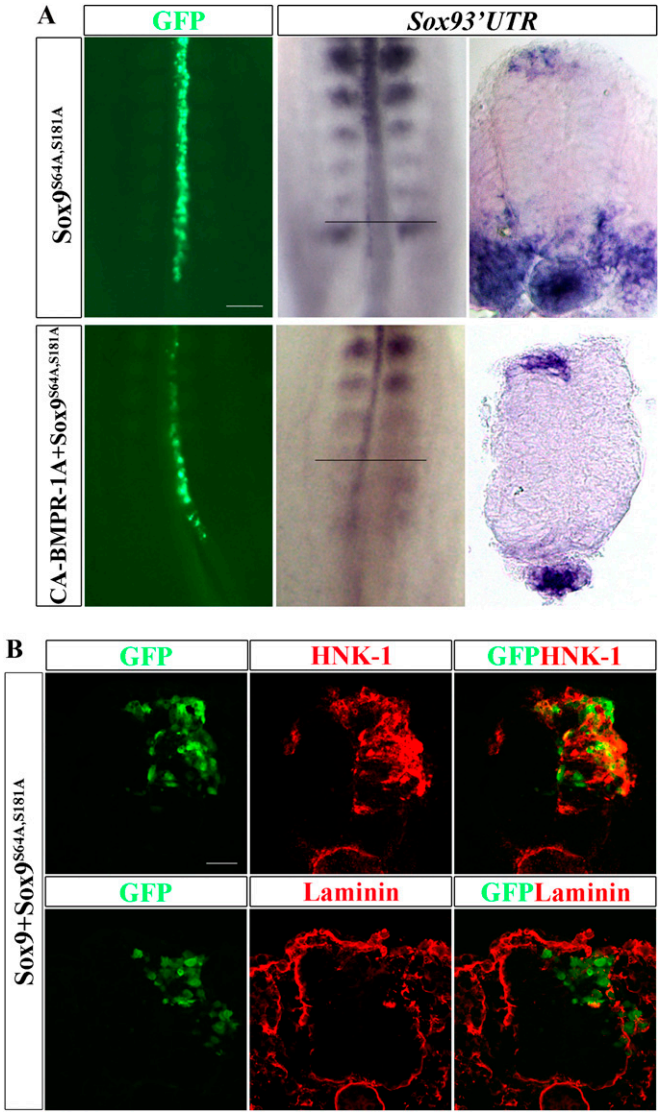

Fig. S2. *Sox9*^{S64A,S181A} does not cause a dominant-negative effect on NC induction and delamination. (*A*) Electroporation of *Sox9*^{S64A,S181A} alone or together with a constitutively active BMP receptor IA (CA-BMPR-IA) into the right side of HH stage 10–11 chicken embryos at the level of segmental plate mesoderm and analyzed at 12 hpt before NC delamination by in situ hybridization with DIG-labeled RNA probe against *Sox9* 3′UTR to detect endogenous *Sox9* expression. Embryos transfected with the indicated constructs result in down-regulation of *Sox9* expression in the premigratory NC region. Black lines indicate the plane of sectioning across the transfected embryos. (*B*) Coelectroporation of WT-*Sox9* and *Sox9*^{S64A,S181A} induces HNK-1 expression and cells emigrating from the dorsal neural tube by down-regulating basal laminin expression. [Scale bars, (*A*) 100 and (*B*) 50 μm.]

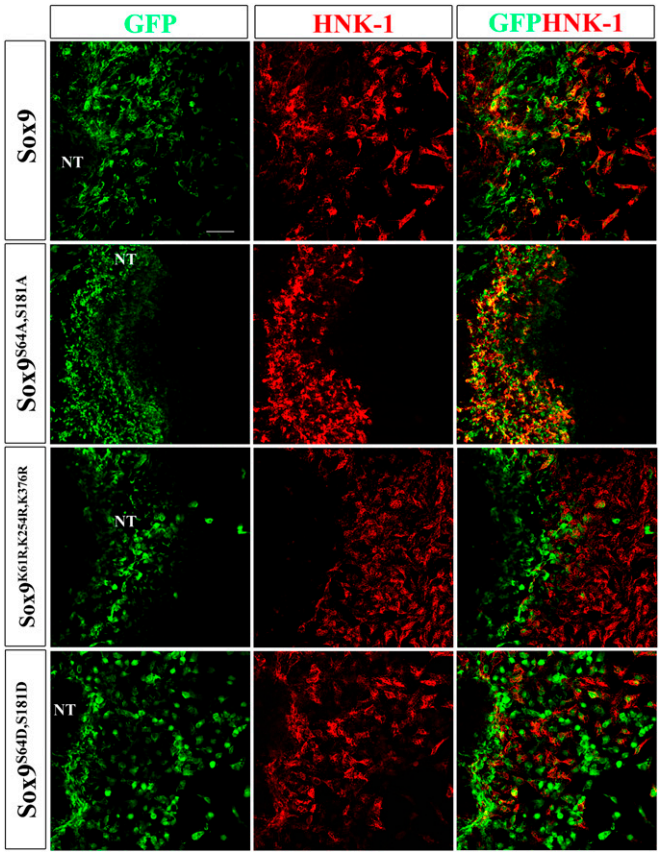

Fig. S3. Phosphorylation of *Sox9* is required for NC delamination in trunk neural explants. HH 11–12 chicken embryos were electroporated with the indicated constructs, harvested at 2 hpt, and excised the neural tubes corresponding to the recently formed somites. Neural tubes were explanted onto fibronectin-coated eight-well slides for 18–24 h before being processed for immunofluorescence with anti-GFP and anti–HNK-1. Misexpression of *Sox9*, $Sox9^{S64D,S181D}$, or $Sox9^{K61R,K254R,K376R}$ induces emigration of NC cells (NCCs) from the neural tube explants, whereas the majority of cells transfected with $Sox9^{S64A,S181A}$ remained in the neural tube. (Scale bar, 50 μm.)

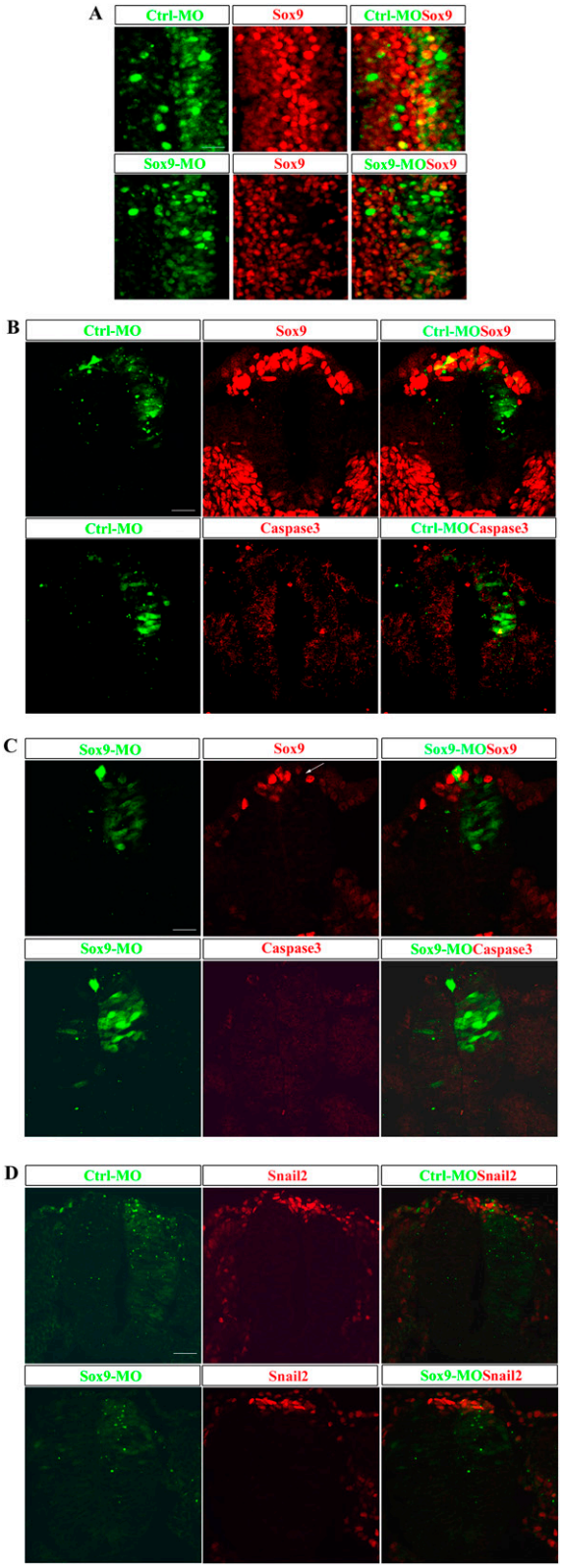

Fig. S4. Ablation of *Sox9* protein in the premigratory NC domain does not lead to apoptosis nor does it affect NC specification. (*A*) In ovo electroporation of fluorescein-tagged Ctrl-MO into the right side of the neural tube has no effect on *Sox9* protein expression along the dorsal midline at 24 hpt, whereas *Sox9* protein expression is lost in the region transfected with *Sox9*-MO compared with the untransfected side (*Left*). (*B*) Transverse section of a Ctrl-MO–treated embryo shows no effect on endogenous *Sox9* protein expression in the premigratory and emigrating NCCs, and no caspase 3–positive apoptotic cells are observed in the transfected side. (*C*) In *Sox9*-MO–treated embryos, *Sox9* protein expression is lost in the premigratory NC region (white arrow), whereas caspase 3–positive apoptotic cells are barely detectable. (*D*) The NC specifier gene, *Snail2*, remains unaltered in both Ctrl-MO– and *Sox9*-MO–treated embryos. [Scale bars, (*A*) 50 and (*B–D*) 100 μm.]

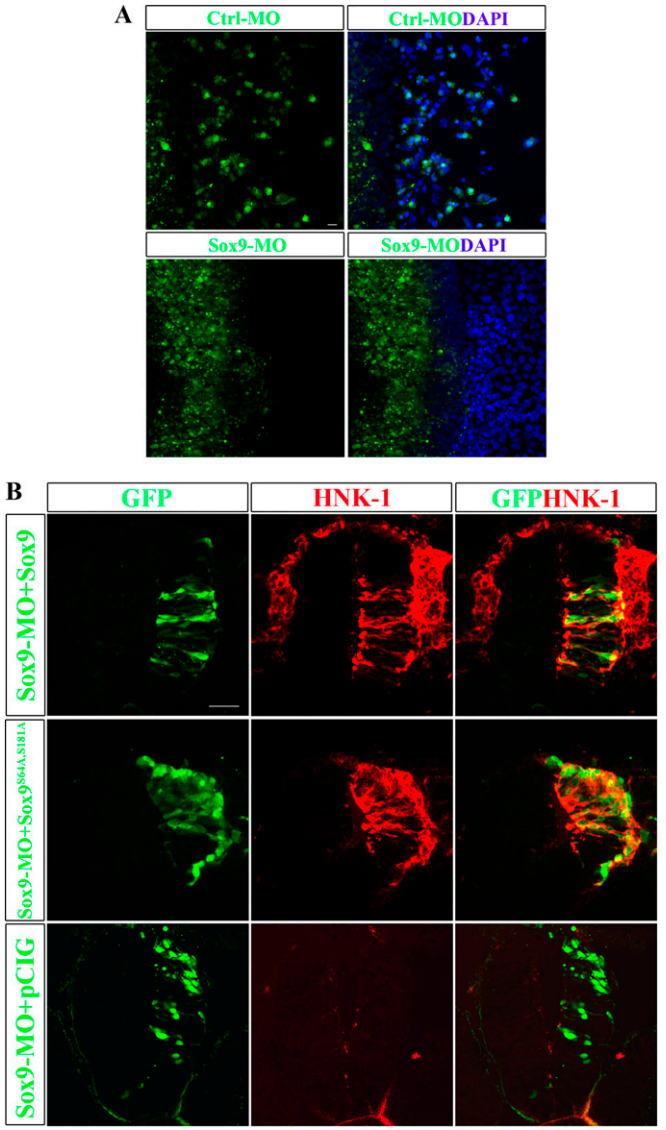

Fig. S5. *Sox9* knockdown prevents NC emigration. (*A*) DAPI stained on neural tube explant shows majority of the cells expressing *Sox9*-MO remain in the neuroepithelium ($n = 6/7$) compared with the Ctrl-MO–treated explant where NC emigration is observed ($n = 7/7$). (*B*) Coelectroporation of *Sox9* or $Sox9^{S64A,S181A}$ plus *Sox9*-MO is able to induce ectopic HNK-1 expression, whereas coelectroporation of *Sox9*-MO plus empty vector (pCIG) does not induce HNK-1 expression. [Scale bars, (*A*) 200 and (*B*) 100 μm.]

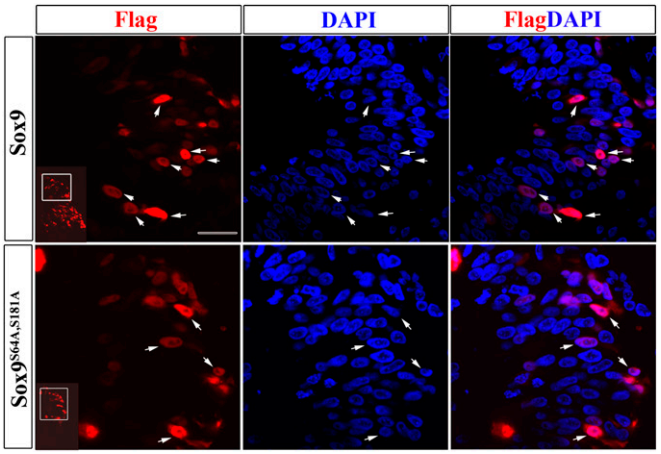

Fig. S6. Mutation of two key phosphorylation residues does not affect nuclear localization of *Sox9* in the developing chick neural tube. Immunofluorescence on the transfected neural tube at 24 hpt with anti-Flag shows ectopic expression of WT-*Sox9* as a positive control and *Sox9*^{S64A,S181A} and DAPI to counterstain for nuclei. Both WT-*Sox9* and *Sox9*^{S64A,S181A} proteins are colocalized with DAPI in the nuclei (white arrows). White box in the *Insets* indicate the region of the transfected neural tube being magnified for illustration. (Scale bar, 50 μm.)