Supplemental Experimental Procedures

Immunohistochemistry, Immunofluorescence and Histology

Mouse embryos or heads were dissected in a cold PBS solution, and fixed from one to four hours in 4% para-formaldehyde while protected from light. After fixation, the tissues were dehydrated through an ethanol gradient (70% for over an hour, 80% for over an hour, 95% for over an hour, and 100% overnight), perforated with xylene, embedded in paraffin and with a microtome, sectioned into 7µm thick segments. Tissue morphology was examined by Hematoxylin and Eosin staining (Cao et al., 2013). Sections used for analysis by immunofluorescence and immunohistochemistry were subjected to antigen retrieval by raising them to 95°C in a citrate buffer (10mM, pH 6.0) for 20 minutes. Blocking was performed by incubating the sections with 20% donkey serum in PBS-triton at room temperature for 30 minutes. Primary antibodies against Sox2 (Goat, R&D Systems, AF2018 1:200; Rabbit Abcam, ab97959, 1:200), GFP (Abcam ab290, 1:500), Ki67 (Abcam, ab15580, 1:200), Lef-1 (Cell signaling #2230, 1:200), , Cleaved caspase-3 (Cell signaling, #9661, 1:200) and amelogenin (Santa Cruz, L0506, 1:200) were then added to the sections. Incubation with primary antibody occurred overnight at 4°C. The slides were treated with FITC (Alexa-488)- or Texas Red (Alexa-555)-conjugated secondary antibody for 30 minutes at room temperature for detection (Invitrogen,1:400). Nuclear counterstaining was performed using DAPI-containing mounting solution. For immunohistochemistry, standard protocols were followed according to the manufacturer's manual (Millipore, IHC select HRP/DAB, DAB150). Images were captured by a Nikon eclipse 80i fluorescence microscope or Zeiss 700 confocal microscope.

BrdU labeling

Two hours prior to sacrifice, pregnant mice were injected with BrdU (10µl/g body weight, Invitrogen, 00-0103), and the embryos were collected and processed as previously described in the immunofluorescence assay. Sections were mounted and rehydrated through a reverse ethanol gradient, and to compensate for endogenous peroxidase activity, immersed in 3% hydrogen peroxide. Antigen retrieval was performed by immersing sections in 10 mM sodium citrate solution for 20 min at a slow boiling state. Sections were perforated by a 30 minute incubation in 2 M HCl, followed by a neutralization step (10 minutes in 0.1M $Na_2B_4O_7$). Sections were

subsequently blocked for 1 hr in 10% donkey serum, and labeled with anti-BrdU antibody (Abcam ab6326, 1:250). Next, standard immunohistochemistry staining was carried out as described above for the immunohistochemistry assays. Experimental and control sections were processed together on the same slide for identical time periods.

IdU/CldU labeling assay

Standard immunofluorescent detection of IdU/CldU was performed according to previous report with modifications (Tuttle et al., 2010). 24 hours prior to harvesting E 15.5 mouse embryos, pregnant female mice were intraperitoneally injected with 100 µg per gram of body weight of CldU (Sigma, C6891). One hour before harvesting the embryos, the pregnant female mice were again injected with 100 µg per gram of body weight of IdU (Sigma, I7125). Mouse embryos were then harvested and embedded in paraffin. The blocks were sectioned and subjected to antigen retrieval by boiling in citrate buffer (10mM, pH 6.0) for 20 min. Sections were then perforated by incubating in 1.5 M HCl at 37°C for 30 minute, followed by a neutralization step (10 minutes 0.1M $Na_2B_4O_7$ at room temperature). Next, sections were blocked in 10% donkey serum diluted in PBST (PBS with 0.05% Triton-100) for one hour at room temperature. Slides were treated with a primary antibody, mouse anti-BrdU/IdU (Roche, 11170376001,1:250), overnight at 4°C to detect IdU. Slides then were stringently washed by vigorous agitation in a shaker for 20 min with low-salt TBST buffer (36mM Tris, 50mM NaCl, 0.5% tween-20; pH 8.0) at 37°C, at a speed of 200 rpm. Sections were washed twice with PBST (10 minutes each), and treated with a primary antibody against CldU (anti BrdU/CldU, Accurate chemical, OBT0030, 1:250) for 2hr at room temperature. Slides were washed three times with PBST, treated with a mix of secondary antibodies (Rhodamine-Red donkey anti-rat, Jackson ImmunoResearch, #712-296-153,1:400; Alex Fluor488 donkey anti-mouse IgG, Invitrogen, A21202, 1:400) for half an hour at room temperature, and then washed three times in 1x PBST for 10 minutes. Slides were covered using a mounting solution containing DAPI (Vector lab, H-1200) and prepared for imaging.

Supplemental References

Cao, H., Jheon, A., Li, X., Sun, Z., Wang, J., Florez, S., Zhang, Z., McManus, M.T., Klein, O.D., and Amendt, B.A. (2013). The Pitx2:miR-200c/141:noggin pathway regulates Bmp signaling and ameloblast differentiation. *Development* **140**, 3348-3359.

Tuttle, A.H., Rankin, M.M., Teta, M., Sartori, D.J., Stein, G.M., Kim, G.J., Virgilio, C., Granger, A., Zhou, D., Long, S.H., et al. (2010). Immunofluorescent detection of two thymidine analogues (CldU and IdU) in primary tissue. *J. Vis. Exp.* **46**, 2166.

Table S1: List of the primers for genotyping.

Mouse lines	Genotyping primers
Pitx2^{Cre}	Cre-Forward: GCATTACCGGTCGATGCAACGAGTGATG Cre-Reverse: GAGTGAACGAACCTGGTCGAAATCAGTGC
Krt14^{Cre}	Same as Pitx2-Cre
Shh^{Cre}	Shh-cre Forward: TGCCAGGATCAGGGTTTAAG Shh-cre Reverse: GCTTGCATGATCTCCGGTAT
Rosa26 *CreERT*	Rosa26-Cre Forward: AAAGTCGCTCTGAGTTGTTAT Rosa26-Cre Reverse: CCTGATCCTGGCAATTTCG
Sox2$^{F/F}$	Sox2Flox mut F:CAGCAGCCTCTGTTCCACATACAC Sox2Flox mut R: CAACGCATTTCAGTTCCCCG Sox2Flox WT F: GCTCTGTTATTGGAATCAGGCTGC Sox2Flox WT R: CTGCTCAGGGAAGGAGGGG
Lef-1$^{cKI/cKI}$	Lef-1cKI mut F: TGAGGCGGAAGTTCCTATTCT Lef-1cKI mut R: GGCGGATCACAAGCAATAAT Lef-1cKI WT F: TCCCAAAGTCGCTCTGAGTT Lef-1cKI WT R: GGCGGATCACAAGCAATAAT
Rosa26$^{Tomato-}$ *GFP*	Tomato-GFP mut F:CTCTGCTGCCTCCTGGCTTCT Tomato-GFP mut R:CGAGGCGGATCACAAGCAATA Tomato-GFP WT F: CTCTGCTGCCTCCTGGCTTCT Tomato-GFP WT R:TCAATGGGCGGGGGTCGTT

Table S2: Primer list for ChIP assay and Real-time PCR.

Primer name	Forward primer(5'-3')	Reverse primer(5'-3')
Pitx2 ChIP 1	AGGGCTGGGAGAAAGAAGAG	ATCTGGCGGAGAATAGTTGG
Pitx2 ChIP 2	GAGCTTCTTTCCGTTGATGC	TTCCCTACTCCACCAACCTG
Pitx2 ChIP control	GGCAGAGTTGGGGTAGATGA	CCCCGTCTAAGTTTCCTTCC
Sox2 ChIP	GCTCAACCTTTGCTCTGGTC	TAGTCCACCCCTCTCACTGC
Sox2 ChIP control	GCCTGGCTCCAATGTAATGT	CATTCCGAGGAAGAGCAGAC
Lef-1	TCACTGTCAGGCGACACTTC	ATGAGGTCTTTTGGGCTCCT
Sox2	ATGCACAACTCGGAGATCAG	TGAGCGTCTTGGTTTTCCG
Pitx2	CTGGAAGCCACTTTCCAGAG	AAGCCATTCTTGCACAGCTC
β - Actin	GCCTTCCTTCTTGGGTATG	ACCACCAGACAGCACTGTG

Supplementary Figures

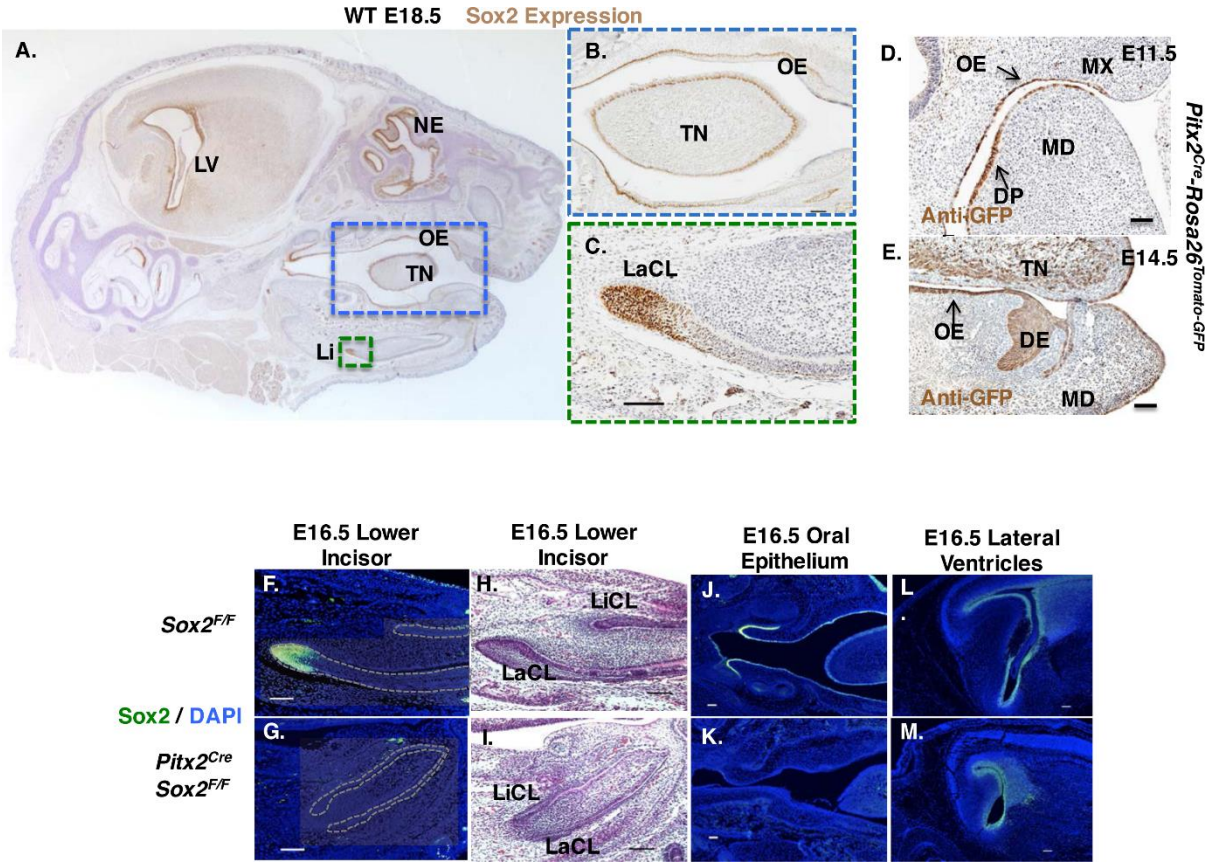

Figure S1. ***Sox2*** **expression in the dental epithelial stem cell niche (LaCL) and loss of Sox2 expression in the dental and oral epithelia in the** ***Pitx2*** ^{Cre}***/Sox2*** $^{F/F}$ **(*Sox2*** ^{cKO}**) embryos. A)** Immunohistochemistry staining of sagittal sections from a WT E18.5 head using a Sox2 antibody reveals that Sox2 is expressed in the lateral ventricle (LV), nasal epithelium (NE), tongue epithelium (TN), oral epithelium (OE) and lower incisor (Li). Nuclei are counterstained with hematoxylin. **B, C)** Magnified views of the blue and green-boxed regions, respectively in (**A**). **B)** Sox2 expression in the OE and TN, **C)** Sox2 expression is localized in the labial cervical loop (LaCL) of the mouse lower incisor. **D, E)** Expression of *Pitx2* ^{Cre} at E11.5 and E14.5, respectively (*Pitx2* ^{Cre} *X Rosa26* $^{tomato\text{-}GFP}$). **F-M)** Immunofluorescence staining of Sox2 in the lower incisor LaCL, oral epithelium and lateral ventricles of *Sox2* $^{F/F}$ (Control) and *Pitx2* ^{Cre}*/Sox2* $^{F/F}$ (*Sox2* ^{cKO}) mice verified the specificity of the deletion of *Sox2* by the *Pitx2* ^{Cre}. Nuclei are counterstained with DAPI. MD, mandible; MX, maxilla; DE, dental epithelium; DP, dental placode, Scale bars, 100µm.

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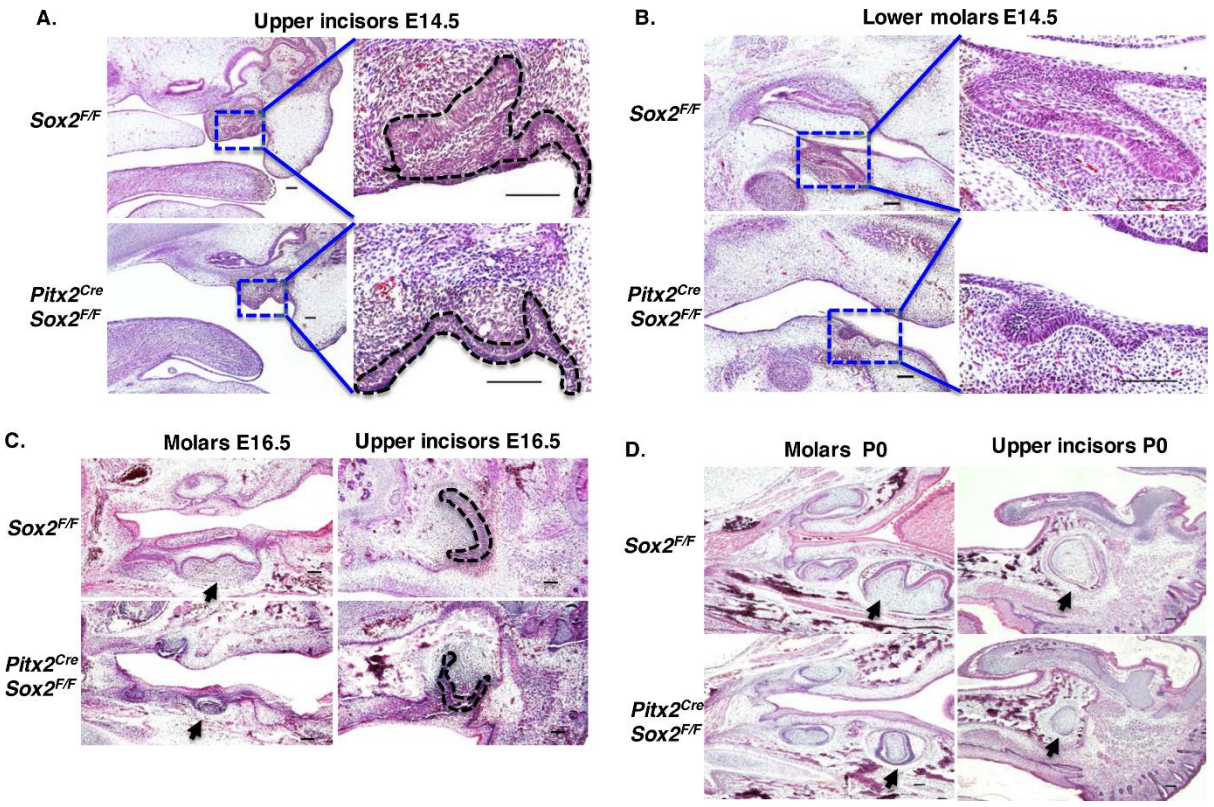

Figure S2. Molar and upper incisor development is impaired in *Sox2*^{cKO} embryos.

A) H&E staining of sagittal section E14.5 upper incisors (UI) shows that *Sox2*^{cKO} UI are smaller with incomplete invagination compared with control (*Sox2*$^{F/F}$). Boxed areas are shown as magnified images on the right (Dotted lines indicate tooth buds). **B)** E14.5 lower molars (LMs) (sagittal section) stained by H&E indicate *Sox2*^{cKO} embryos have a smaller LM with incomplete invagination compared to controls. Boxed areas are shown as magnified images on the right. **C)** H&E staining of E16.5 UI and molars show a smaller size and abnormal shape in *Sox2*^{cKO} embryos compared with controls. Arrows indicate molars and dotted line indicates UI). **D)** P0 molars and upper incisors stained with H&E. *Sox2*^{cKO} molars are smaller and hypoplastic compared with control molars. Upper incisors are also smaller in *Sox2*^{cKO} mice. Arrows indicate molars and UI, respectively). Scale bars, 100µm.

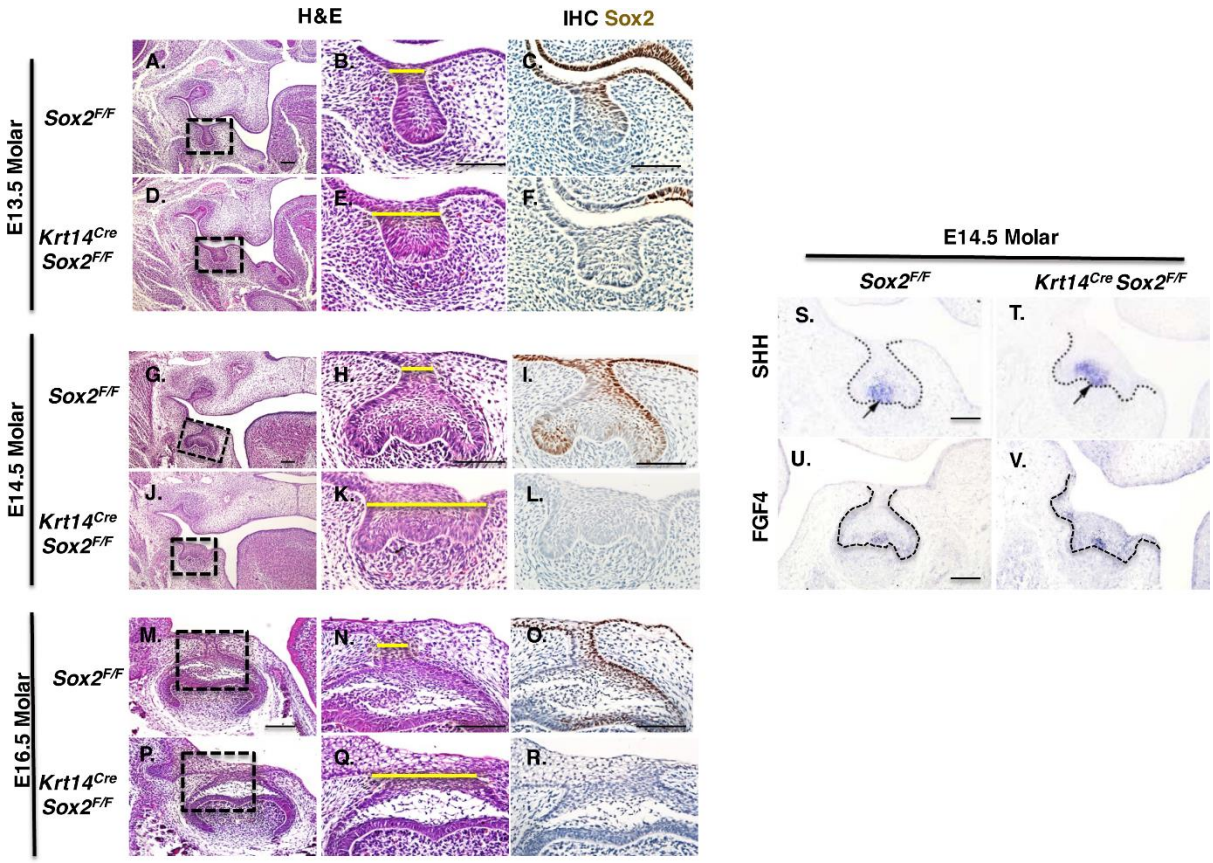

Figure S3. *Sox2* ablation with the *Krt14*^{Cre} causes abnormal molar formation and changes in *Shh* and *Fgf4* expression.

A-F) At E13.5, invagination of the lower molar tooth bud was displaced with a wide dental lamina and tooth bud structure (yellow line) in the *Krt14*^{Cre}/*Sox2*$^{F/F}$ (E, F), compared to controls (B, C). **G-L)** At E14.5, invagination was affected and the dental lamina was significantly larger in *Krt14*^{Cre}/*Sox2*$^{F/F}$ embryos compared to controls. **M-R)** At E15.5, the invagination defect persisted with abnormal formation of the molar in the *Krt14*^{Cre}/*Sox2*$^{F/F}$ embryos. Interestingly, in the incisors Sox2 was asymmetrically expressed (C, I, O). **S, T)** *Shh* transcripts were detected in the *Sox2*$^{F/F}$ enamel knot structure of the E14.5 lower molar, however the *Shh* expression domain was expanded in the *Krt14*^{Cre}/*Sox2*$^{F/F}$ lower molar, as the *Shh* domain moved towards the presumptive Sox2 region. **U, V)** *Fgf4* expression (transcripts) was slightly increased in the *Krt14*^{Cre}/*Sox2*$^{F/F}$ lower molars.

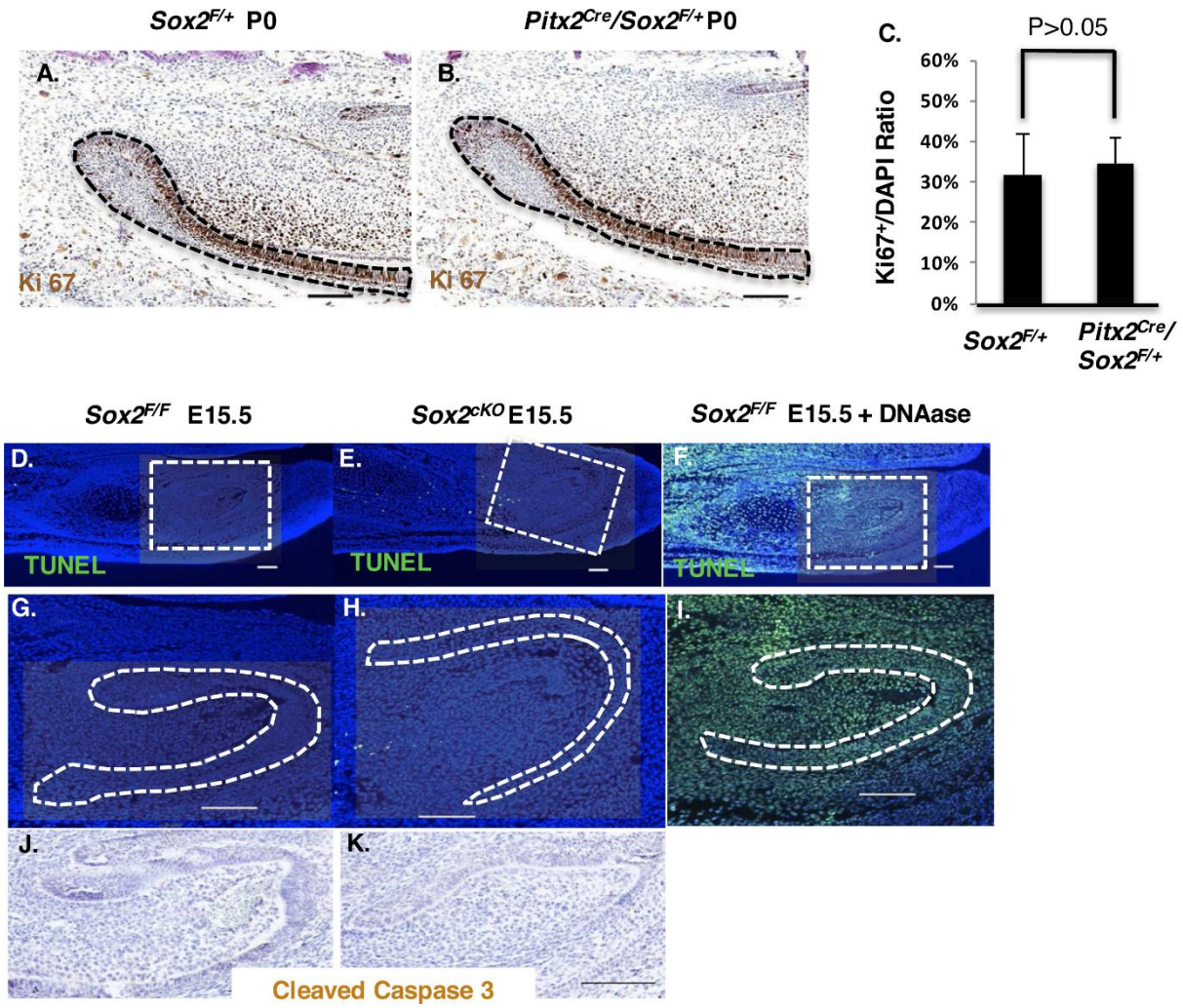

Figure S4. Loss of dental epithelial stem cells in *Sox2*^{cKO} embryos does not involve increased apoptosis.

A-B) Control experiments show that proliferation of the dental epithelial cells in the LaCL was not affected in the P0 *Sox2*$^{F/+}$ or *Pitx2*^{Cre}/*Sox2*$^{F/+}$ embryos. **C)** Quantitation of the Ki67 stained cells in the *Pitx2*^{Cre}/*Sox2*$^{F/+}$ embryos and controls shows no difference in proliferation between groups. **D-I)** TUNEL staining in E15.5 (sagittal section) mouse lower incisors reveals no changes in apoptosis in *Sox2*^{cKO} embryos. Magnified view of boxed regions in D, E and F are shown in G, H and I respectively. No apparent apoptosis signal was detected in control or *Sox2*^{cKO} incisors. (F, I) are positive controls for TUNEL staining. N=3. Nuclei are counterstained with DAPI **J, K)** Immunohistochemistry staining of the early apoptosis marker cleaved Caspase 3 in sagittal sections of E15.5 mouse incisors showing no detectable level of apoptosis in control and *Sox2*^{cKO} incisors, N=3. Nuclei are counterstained with hematoxylin. Scale bars, 100μm.

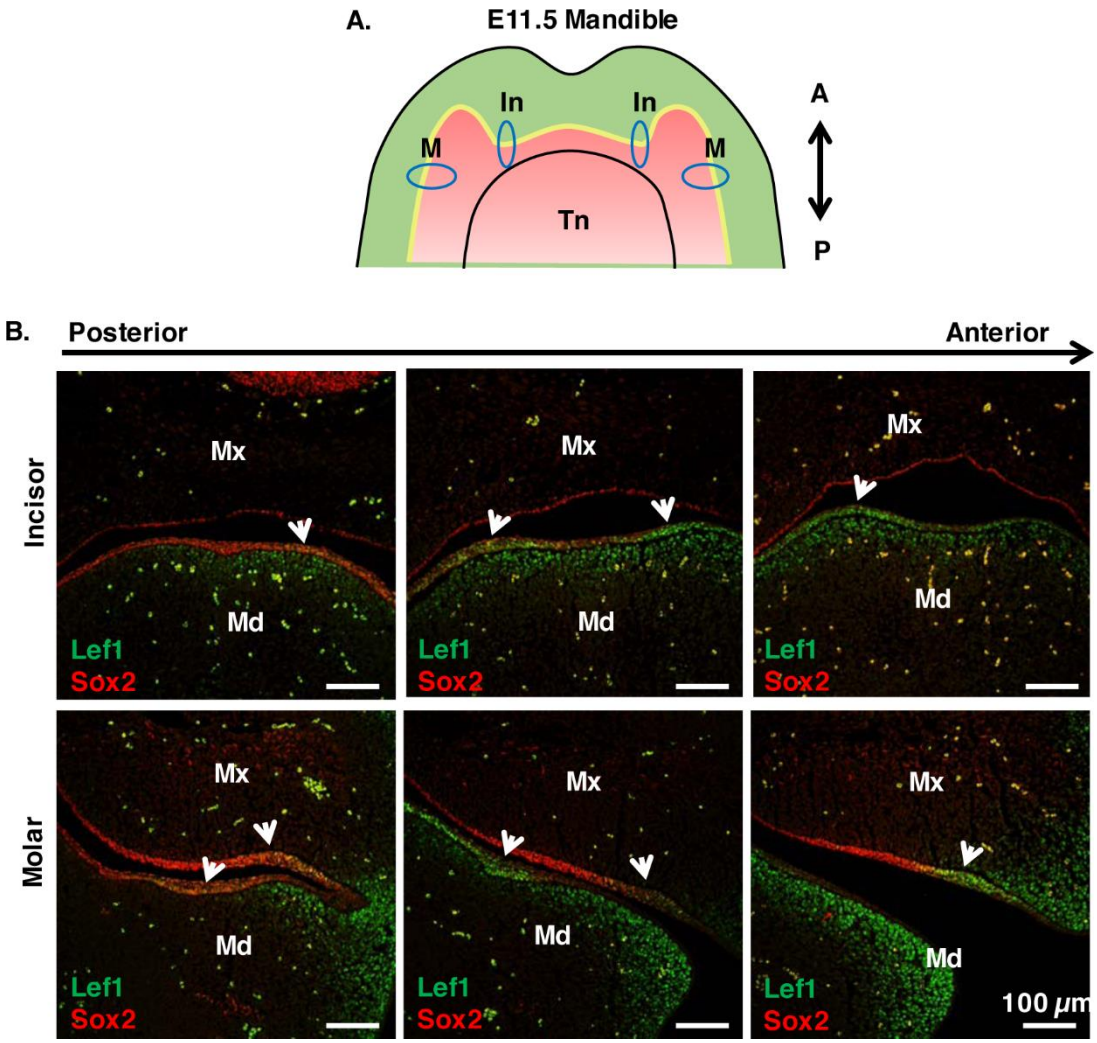

Figure S5. Sox2 expression is posterior to Lef-1 in developing incisors and molars.

A) Schematic of the murine E11.5 mandible showing Sox2 expression in red and Lef-1 expression in green. The locations of the incisors (In) and molars (M) are shown by blue circles (A, anterior; P, posterior; Tn tongue). **B)** Merged photos of Sox2 and Lef-1 immunofluorescence staining in E11.5 WT embryos from posterior to anterior showing the juxtaposed expression domains of these two factors. Arrows denote the dental placodes. Mx, maxilla; Md, mandible. Scale bars, 100μm.

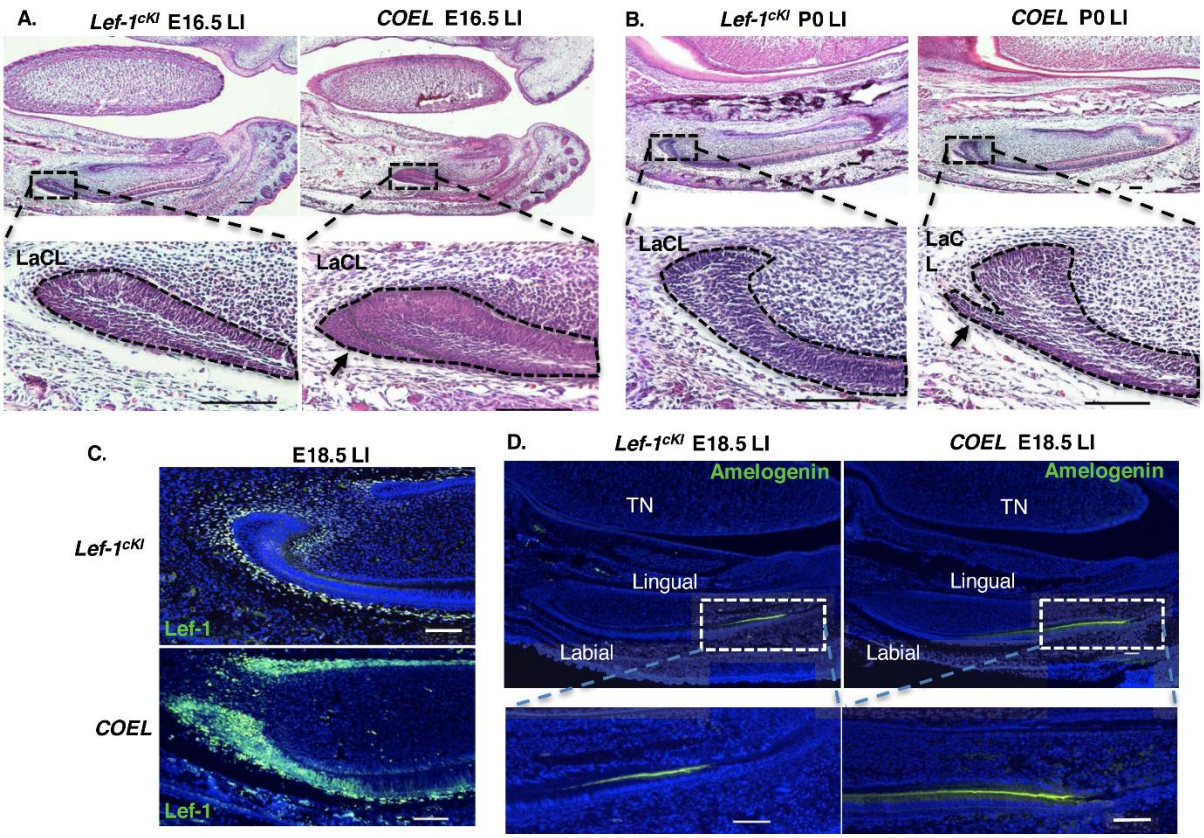

Figure S6. Overexpression of *Lef-1* in dental and oral epithelia creates a new LaCL stem cell compartment and increased amelogenin expression.

A, B) H&E staining of lower incisors (sagittal sections) shows an enlarged LaCL in E16.5 *COEL* incisors compared to controls (*Lef-1*^{cKI}). The arrow denotes the separation of a new stem cell compartment from the normal cervical loop structure in the *COEL* LI. A newly formed stem cell compartment (arrow) that has branched from the LaCL was observed in P0 *COEL* incisors (N=3). **C)** Lef-1 immunofluorescence staining in E18.5 lower incisors shows Lef-1 was overexpressed in the LaCL and LiCL of the COEL embryos. **D)** Amelogenin immunofluorescence staining in E18.5 lower incisors indicates increased amelogenin expression in *COEL* incisors compared to control incisors. Nuclei are counterstained with DAPI. Tn, tongue. Scale bars, 100μm.

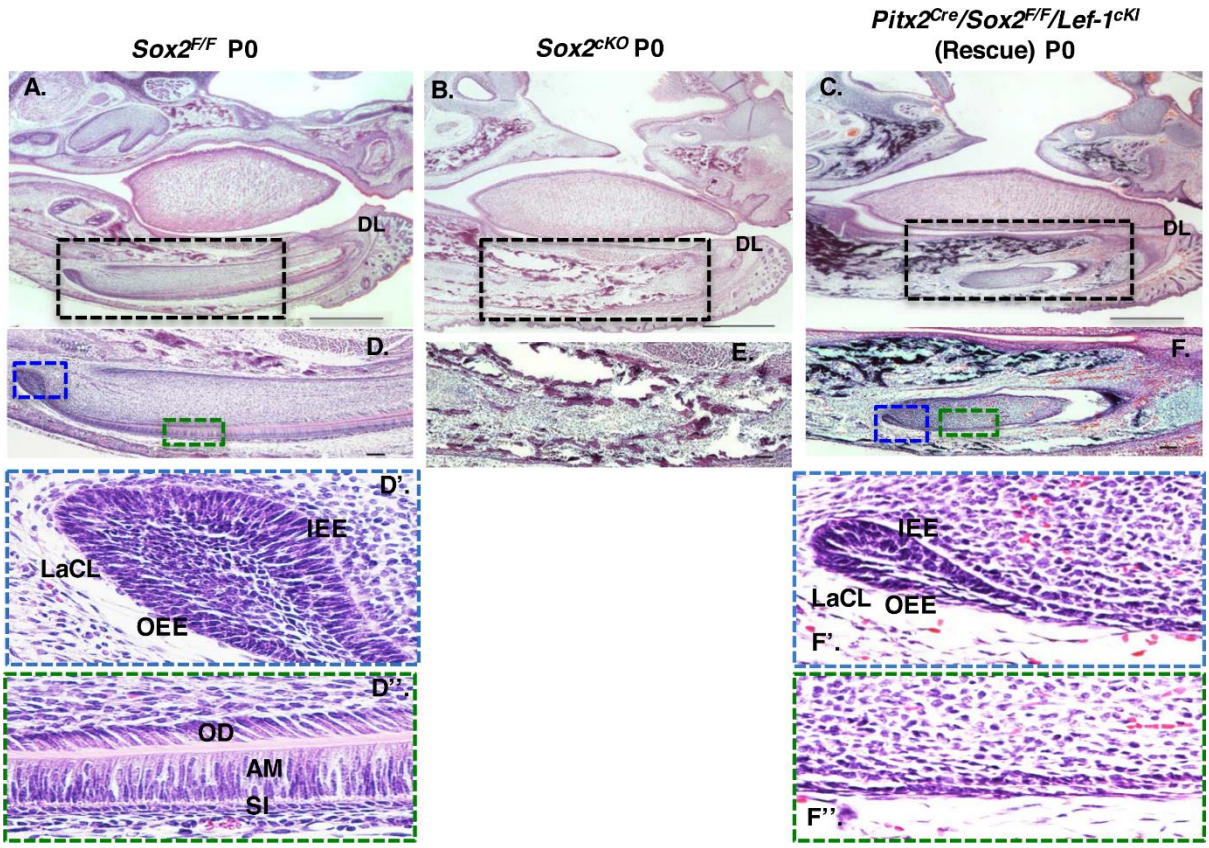

Figure S7. *Lef-1* overexpression rescues lower incisor development in *Sox2*^{cKO} embryos.

A-C) H&E staining (sagittal sections) of P0 *Sox2*$^{F/F}$ (control), *Sox2*^{cKO} and *Pitx2*^{Cre}/*Sox2*$^{F/F}$/*Lef-1*^{cKI} (*Lef-1* rescue *Sox2*^{cKO}) embryos. **D-F)** Higher magnification of black boxed regions in A, B and C show their respective lower incisors. Note the absence a lower incisor in the *Sox2*^{cKO} mice (E). Lower incisors were observed in rescue mice (F). **D', F')** Higher magnification of the blue-boxed LaCL (D) shows cells in the stellate reticulum (stem cells) surrounded by the polarized outer and inner enamel epithelium in the control (D'). However, in the rescue mice a LaCL is formed but contains fewer progenitor cells and the outer and inner enamel epithelial cells are not well polarized (blue box, F'). **D'', F'')** Higher magnification of the green box in D and F. The rescue LIs (F'') show a lack of differentiation on the labial side and do not contain the differentiated odontoblasts, ameloblasts tratum intermedium observed in the control incisors (D''). AM, ameloblast; OD, odontoblast; LaCL, labial cervical loop; SI, stratum intermedium; OEE, outer enamel epithelium; IEE, inner enamel epithelium; DL, dental lamina. Scale bars, 100μm.

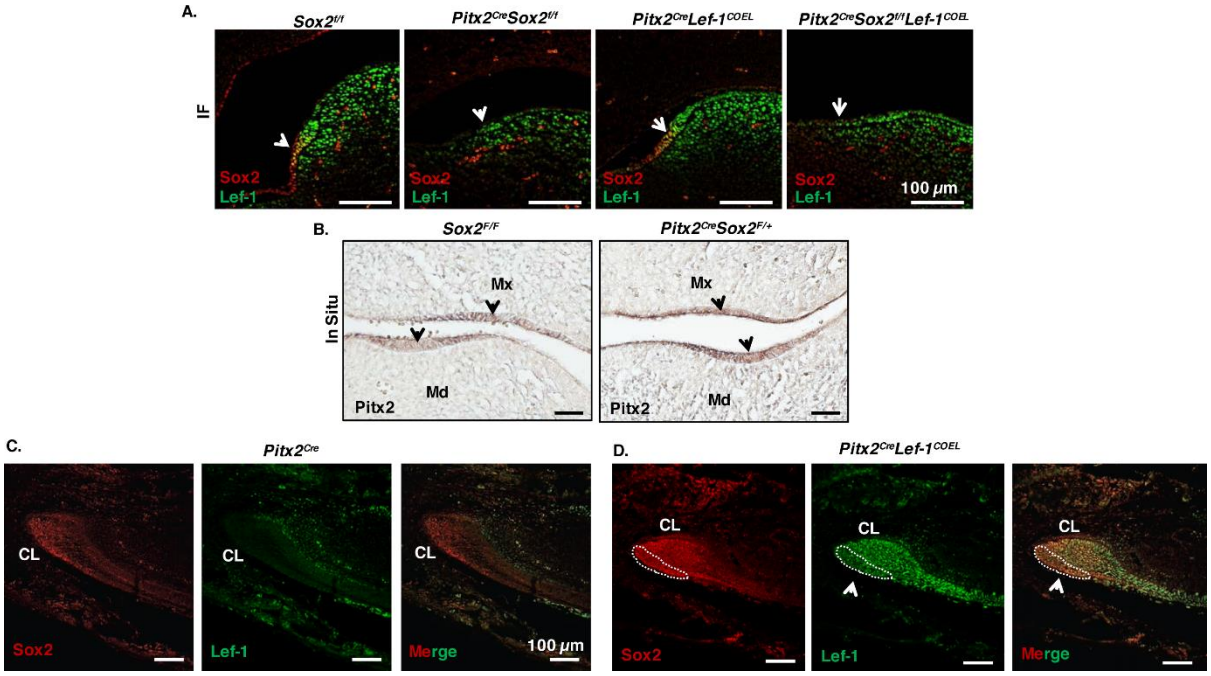

Figure S8. Sox2 and Lef-1 act independently to regulate early incisor development.

A) Merged photos of Sox2 and Lef-1 immunofluorescence staining in E11.5 *Sox2*$^{F/F}$, *Sox2*^{cKO}, *COEL* and rescue embryos, showing the juxtaposed expression domains of these two factors. Loss of *Sox2* does not affect Lef-1 expression, and over expression of Lef-1 does not affect Sox2 expression. Arrows denote the dental placodes. **B)** *Pitx2* in situ hybridization experiments showing *Pitx2* expression in the lower incisor placodes of E11.5 embryos. The arrows denote the incisor epithelial thickenings. **C, D)** Sox2 and Lef-1 immunofluorescence staining in the LaCL of E18.5 *Pitx2*^{Cre} and COEL embryos, respectively. Lef-1 over expression (D) creates a new stem cell compartment (arrow). These cells express Sox2 but have reduced Lef-1 expression compared to Lef-1 expression in the other regions of the LaCL.

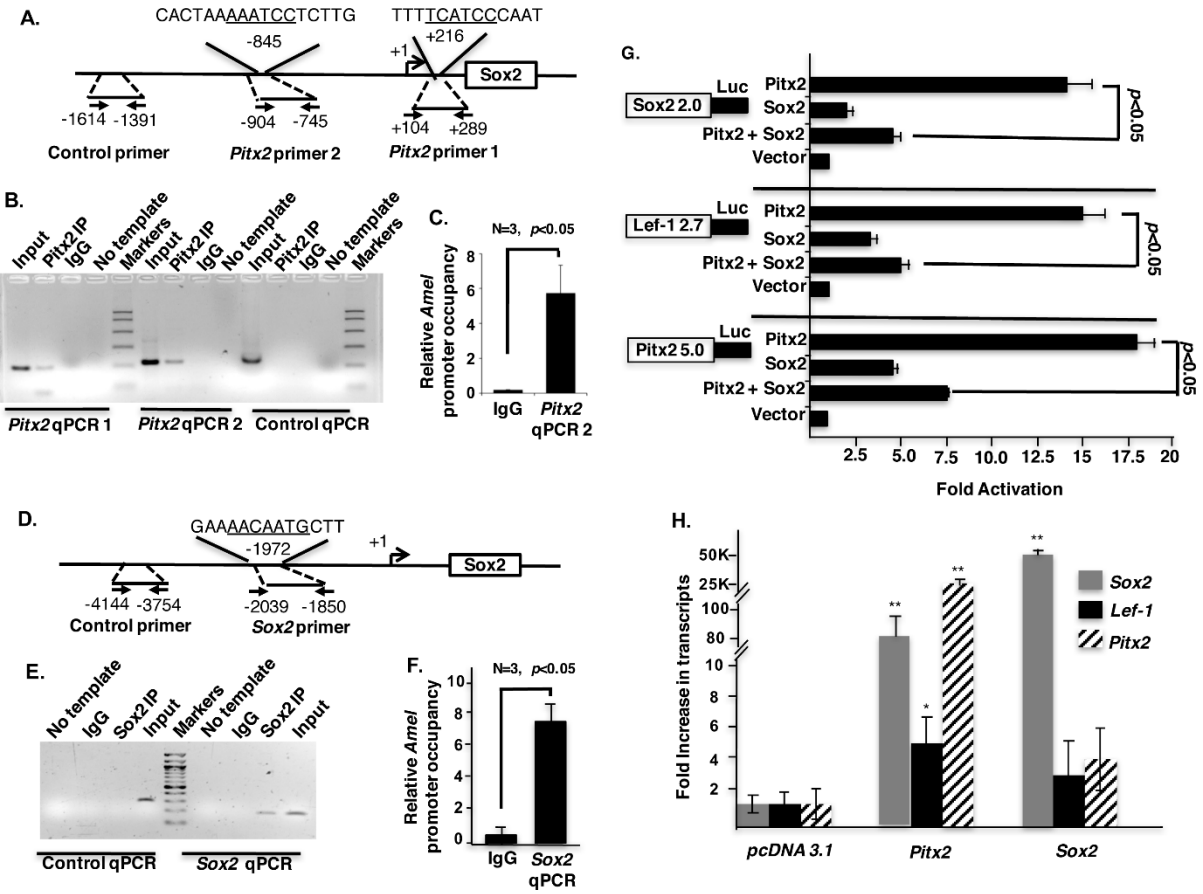

Figure S9. Endogenous Pitx2 and Sox2 bind to elements in the *Sox2* promoter.

A) Schematic of the *Sox2* promoter region displaying two putative Pitx2 binding sites (216bp downstream of the transcriptional start site (TSS) and 845bp upstream of the TSS). The binding sequences are underlined. **B)** ChIP assays were performed on LS-8 cells and PCR products were resolved on agarose gels. Pitx2 antibody (Ab) immunoprecipitated (IP) the chromatin containing the Pitx2 binding sites but not a control site which did not contain a putative Pitx2 binding site. **C)** ChIP-qPCR analyses to assess the enrichment of the binding by Pitx2 Ab compared to IgG using Pitx2 primer 2 probes. **D)** A putative Sox2 binding site was identified at position 1972 upstream of *Sox2* TSS. Primers used to amplify the putative binding site and control site, which doesn't have a putative Sox2 binding, are indicated. **E)** ChIP assays were carried out on LS-8 cells by using a Sox2 Ab and the PCR results demonstrate that endogenous Sox2 binds to this Sox2 binding site in the Sox2 promoter region. **F)** ChIP-qPCR shows an 8-fold enrichment by using Sox2 Ab to pull-down this chromatin region compared to using IgG. **G)** Expression plasmids containing the Pitx2, Sox2, and vector-only cDNAs were co-transfected into LS-8 cells with a luciferase reporter plasmid whose expression was driven by the *Sox2, Lef-1* or *Pitx2* promoter. Luciferase activity is shown as mean-fold activation compared to activation in the presence of empty mock expression plasmid. All luciferase activities were normalized to β-galactosidase expression. **H)** *Sox2, Lef-1* and *Pitx2* transcripts from LS-8 cells transfected with vector only, Pitx2 or Sox2 were assessed by real-time PCR. The *Pitx2*, *Sox2* and *Lef-1* mRNA level in overexpressing Pitx2 and Sox2 cells were compared to overexpressing empty vector control cells. Pitx2 activates endogenous *Sox2* and *Lef-1* expression while overexpression of Sox2 shows no significant changes in endogenous expression levels of either *Lef-1* or *Pitx2* (N=3). All data normalized to β-actin transcripts. *, $p<0.05$. **, $p<0.001$. (Where are the * in panel H? please see the attached new fig S9)

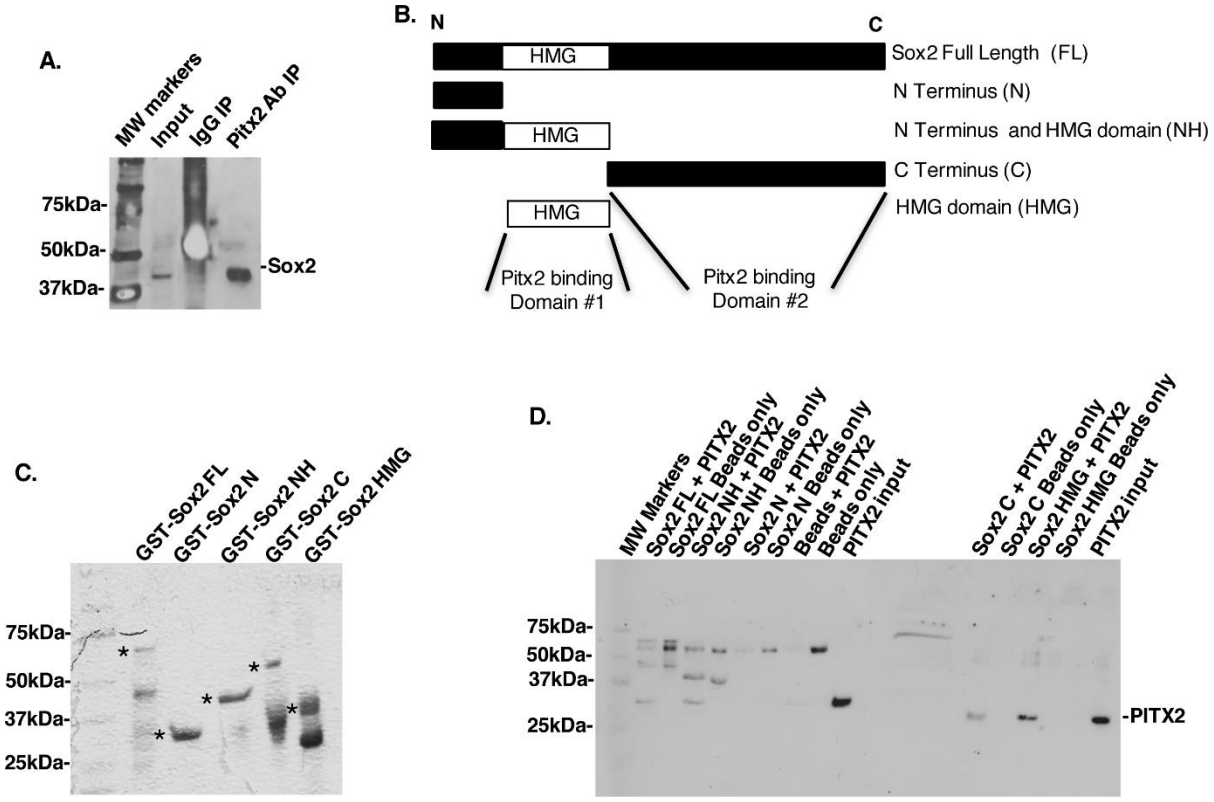

Figure S10. Sox2 interacts with Pitx2 through Sox2 HMG and C-terminal domains.

A) Immunoprecipitation (IP) of endogenous Sox2 using the Pitx2 Ab in LS-8 cells. The input was 5% of the lysate used in the IP assays. IgG used as a control did not IP the Sox2 protein, whereas the Pitx2 Ab did IP the Sox2 protein. **B)** Schematic of the GST-Sox2 truncated constructs used to determine the protein interaction domain of Sox2. The two Pitx2 binding domains are shown. **C)** GST-Sox2 constructs were bacteria expressed, purified and used in the GST-pull down assay. The fusion proteins are shown on a coomassie blue stained SDS PAGE gel. **D)** Purified Pitx2 protein was incubated with the GST-Sox2 constructs to determine which regions of Sox2 bound Pitx2. Pitx2 bound to the HMG and C-Terminal domains of Sox2.