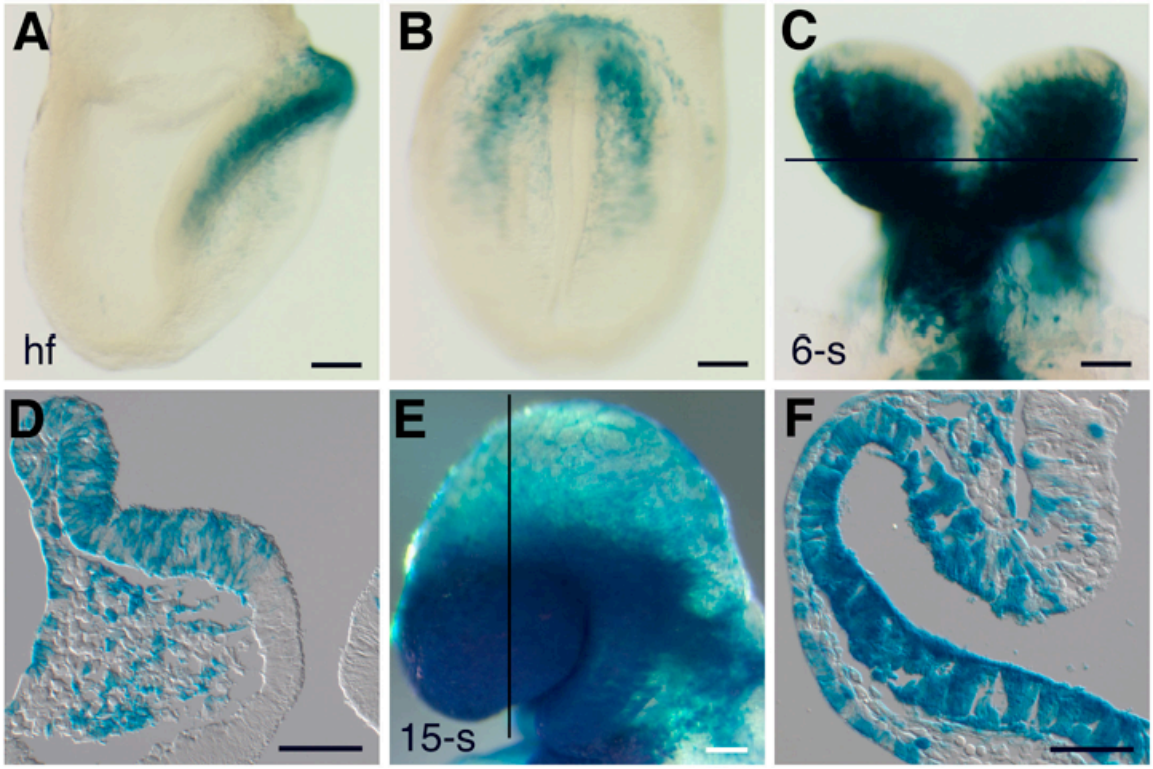
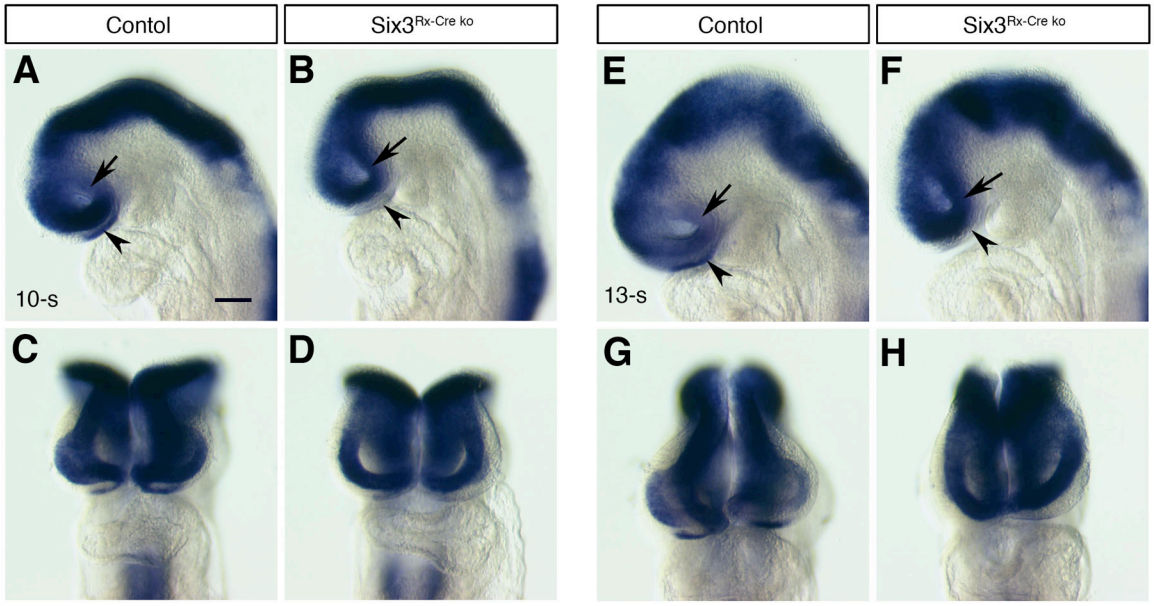
Supplementary Figure 1



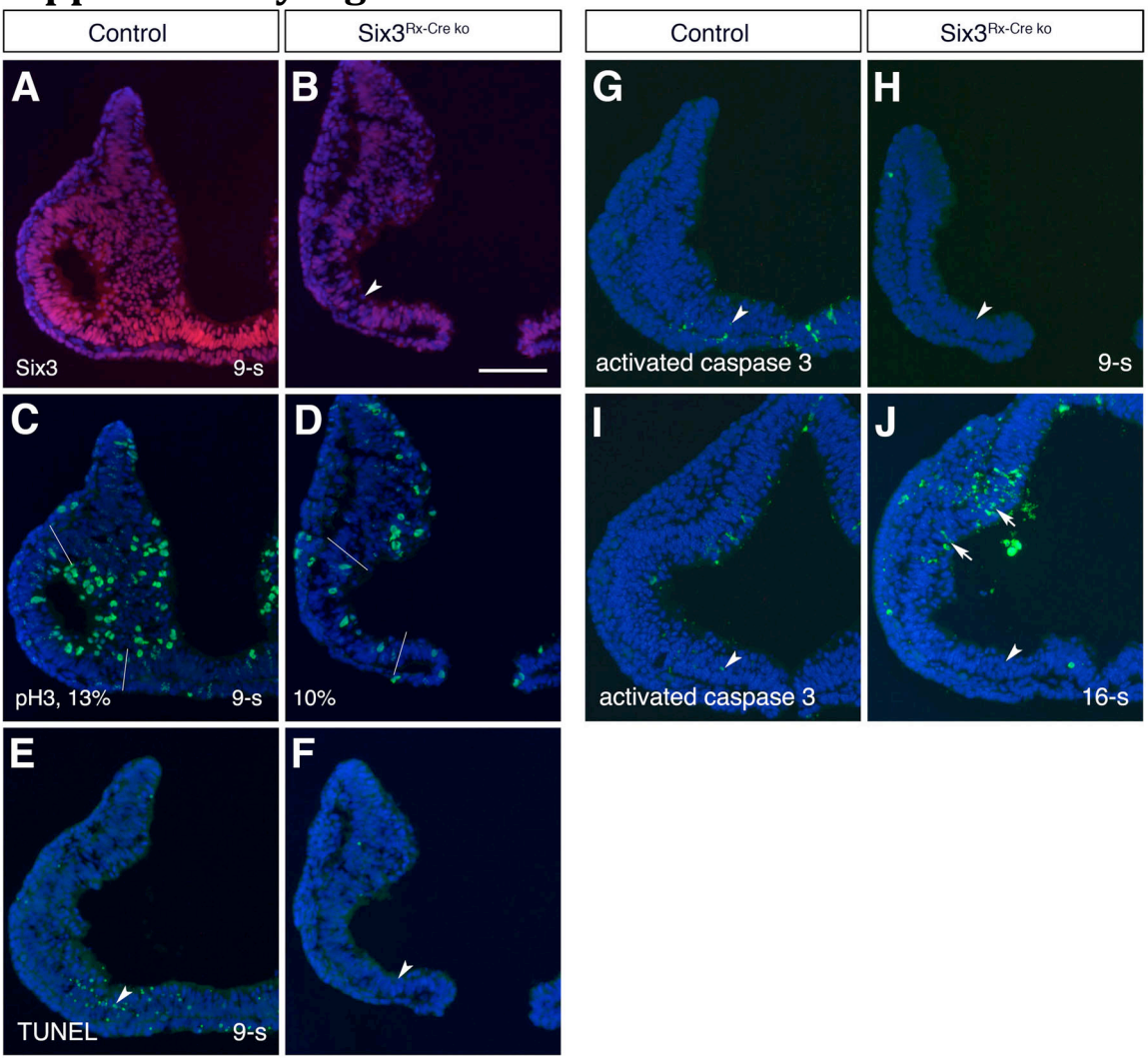
Supp. Figure 1. Rx-Cre is active in the eye field and optic vesicles. To evaluate the temporal and spatial activity of the Rx-Cre transgenic line, we crossed this strain with the R26R reporter mouse line and subjected isolated embryos to X-gal staining. X-gal^{+} cells are detected as early as the head-fold stage in the putative anterior neural plate region (**A**, **B**). At the 6-somite stage, X-gal^{+} cells are seen in the anterior neural plate (**C**), including the eye field territory in the corresponding section (**D**). Soon thereafter, extensive X-gal staining is seen over the entire developing eye (**E**), including the optic vesicle territory (**F**). Scale bar = 100 μm.

Supplementary Figure 2



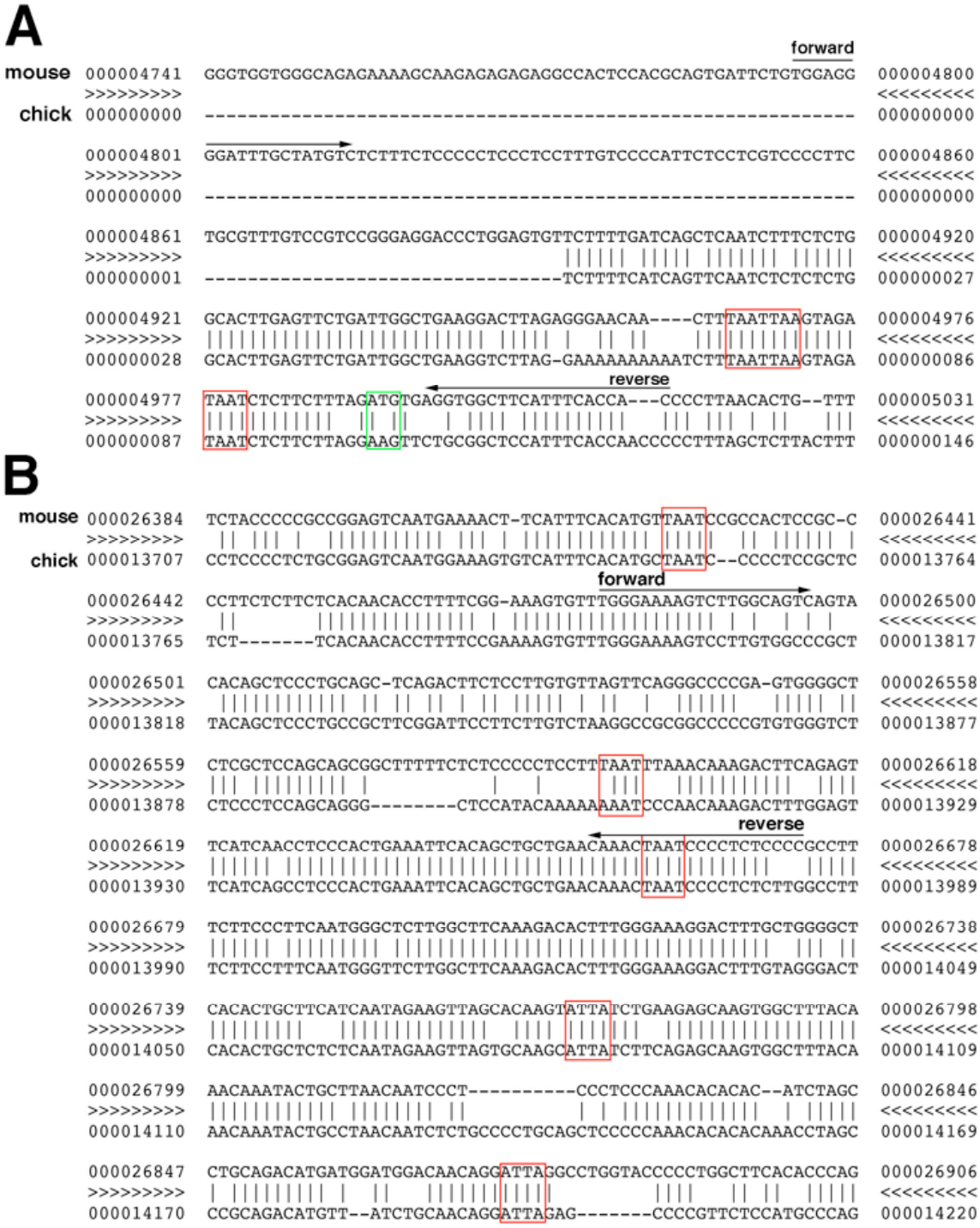
Supp. Figure 2. *Sox2* expression is not affected in *Six3*-deficient optic vesicles. Whole-mount in situ hybridization at the 10-somite (**A**, **C**) and 13-somite (**E**, **G**) stage revealed normal *Sox2* expression in the developing forebrain, including the optic vesicle territory (arrows) and adjacent surface ectoderm (arrowheads). (**B**, **D**, **F**, **H**) *Sox2* expression appears unaffected in *Six3* conditional–mutant littermates, except in the surface ectoderm where its expression appears to be reduced (arrowheads). Scale bar = 100 μm.

Supplementary Figure 3



Supp. Figure 3. No major alterations in cell proliferation or apoptosis are observed in *Six3* conditional–mutant embryos. Coronal sections of 9-somite staged embryos were used for proliferation and TUNEL assays. (**A**) Immunofluorescence antibody staining at this stage shows that *Six3* is expressed in the whole optic vesicle territory of wild-type embryos. (**B**) In mutant littermates, *Six3* expression is absent or significantly reduced from most cells in this region (arrowhead). (**C**, **D**) A slightly lower proliferation index in the mutant optic vesicle territory (between the dashed white lines) was observed after counting cells on serial sections immunostained for pH3. (**E**, **G**) At this stage, few TUNEL^{+} or activated caspase3^{+} cells were observed in the ventral optic vesicle region of control embryos (arrowheads). (**F**, **H**) These cells were not seen in Six3-deficient littermates (arrowheads), and no ectopic apoptosis were observed. (**I**, **J**) At the 16-somite stage, ectopic apoptosis was observed in the dorsal optic vesicle and pallidal neuroepithelium of Six3-deficient embryos (arrows in **J**). Scale bar = 100 µm.

Supplementary Figure 4



Supp. Figure 4. Putative Six3 DNA-binding motifs are present in the promoter and the 3'-flanking region of the chicken and mouse *Wnt8b* genomic locus. Sequences of the area A and B shown in Fig. 5A are included.

Putative Six3-binding motifs are highlighted by red squares in the promoter region (**A**) and the 3'-flanking domain (**B**). The ATG-initiation codon is highlighted in light green. Primers used for EMSA qPCR are also indicated.

Supplemental Methods

ChIP assay and qPCR

Four litters of E8.5 wild-type embryos (46 total embryos between 8- and 15-somites) were harvested and immediately fixed in 10 ml of 1% formaldehyde for 15 minutes at room temperature with agitation. Fixation was stopped by adding 1.25 ml of 1 M glycine for 5 minutes followed by 3 washes in PBS. Heads (Six3-positive) and trunks (Six3-negative) were dissected and pooled separately, and then lysed in 1 ml of Cell Lysis Buffer (5mM PIPES, pH8.0, 85mM KCl, 0.5% NP 40 plus protease inhibitors) by passing through a 25 gauge needle 8 times and incubating for 10 minutes (cell lysis can be monitored under a microscope by Trypan Blue staining). The nuclei were harvested by centrifugation at 3000 rpm for 5 minutes and lysed in Lysis Buffer (20mM Tris-HCl, pH7.5, 1% Triton X100, 1mM EDTA, protease inhibitors). Equal amounts of chromatin from the head and trunk (adjusted to 500µl) were sonicated on ice (15 sec, 15 times, power scale at 2). The sonicated chromatin was diluted with 1ml of Binding Buffer (20mM Tris-HCl, pH7.5, 210mM NaCl, 0.5% Triton X100, protease inhibitors), and pre-cleared by adding 4 µl of normal rabbit IgG (Jackson) for a 30 minute incubation at 4°C. Then 75µl of protein A agarose (Upstate) was added and incubated for an additional 60 minutes incubation at 4°C, and finally was centrifuged at 3000 rpm for 1 minute.

The pre-cleared chromatin (supernatant) was split in half: 4 µl of Six3 antibody was added to one half and 4 µl of normal rabbit IgG was added to the other half, and both were incubated at 4°C overnight. The immuno-bound chromatin was pulled down by adding 60 µl of protein A agarose, incubating for 60 minutes and then centrifuging for 1 minute at 3000 rpm. Supernatant for the IgG-immunoprecipitated sample was saved to use as an input control. The beads, to which immunoprecipitated chromatin was bound, were washed with 1 ml of Wash Buffer (20mM Tris-HCl, pH7.5, 140mM NaCl, 0.5% Triton X100) for 5 minutes at room temperature 5 times. Immunoprecipitated chromatin was eluted from the beads with 250 µl of Elution Buffer (20mM Tris-HCl, pH7.5, 10mM EDTA, 1%SDS). The eluted chromatin was un-crosslinked by adding 20 µl of 5M NaCl and incubating for 5 hours at 65°C, purified by proteinase K treatment for 2 hours at 45°C and phenol/chloroform extracted, and precipitated with ethanol and dissolved in 50 µl of TE.

2 µl of DNA from each of the four samples (Six3 or IgG immunoprecipitated heads or trunks) was used for qPCR (SYBR green) using an absolute quantification protocol (Applied Biosystems). The sequence of the primers is indicated in Sup.5. A series of dilutions of the input sample was used as a standard, and the quantitative value of each sample was calculated based on the standard curve (the value of each ChIP sample should fall within the range of the dilutions). The enrichment was expressed as the ratio of the Six3-immunoprecipitated sample over its corresponding IgG-immunoprecipitated sample.