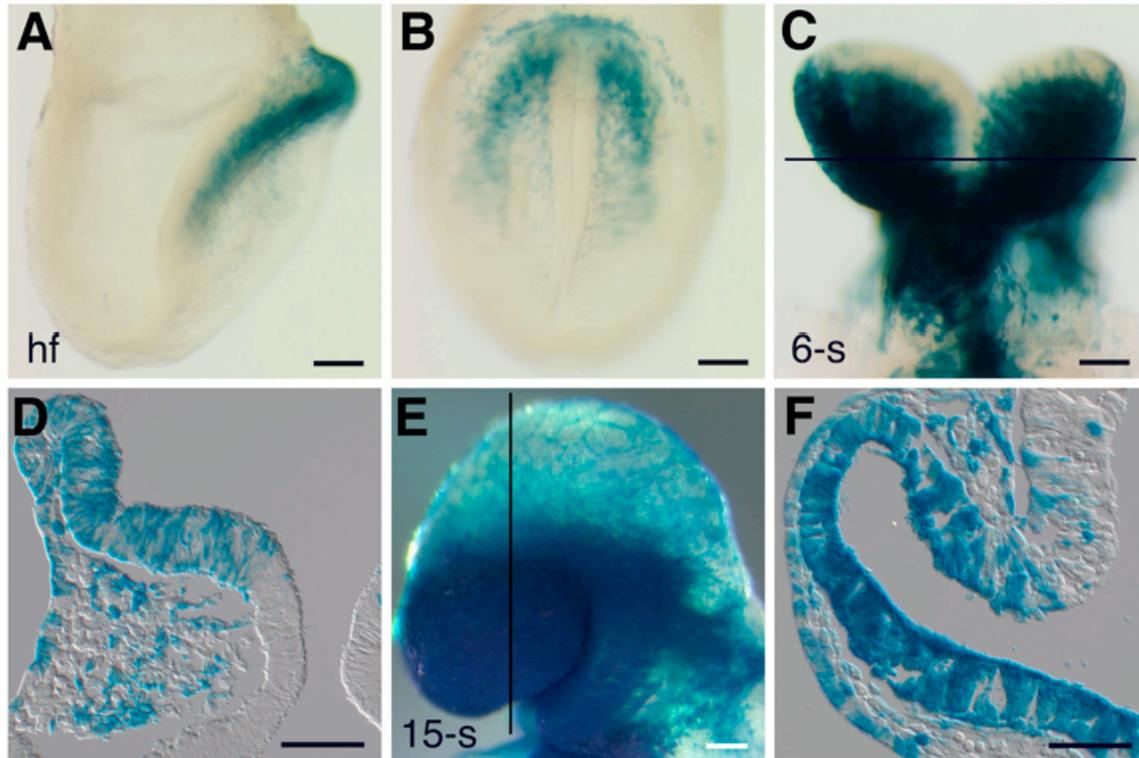
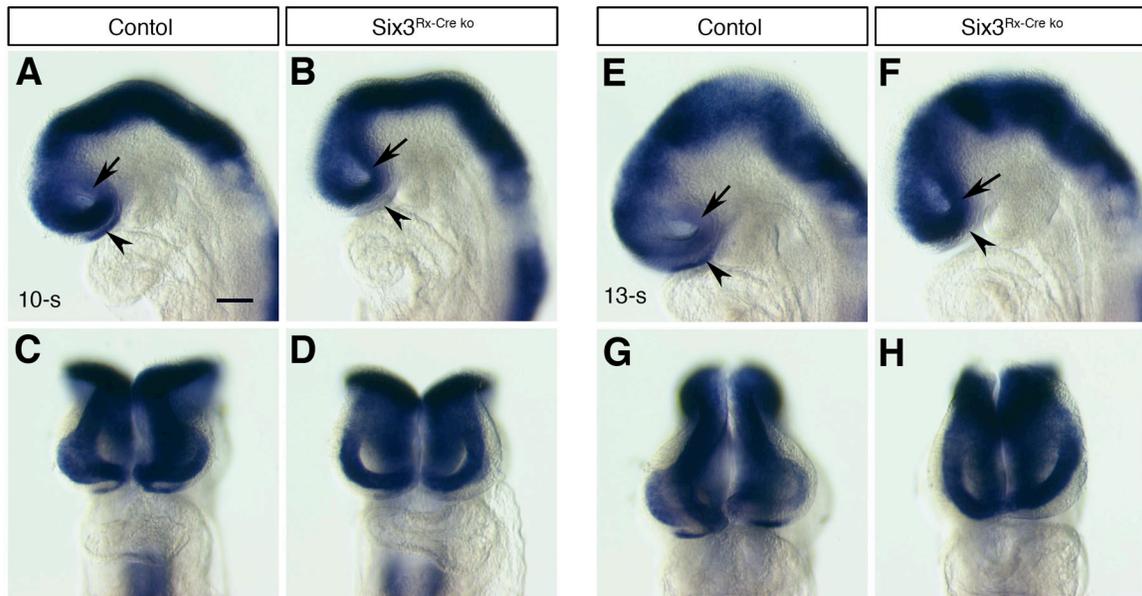


Supplementary Figure 1



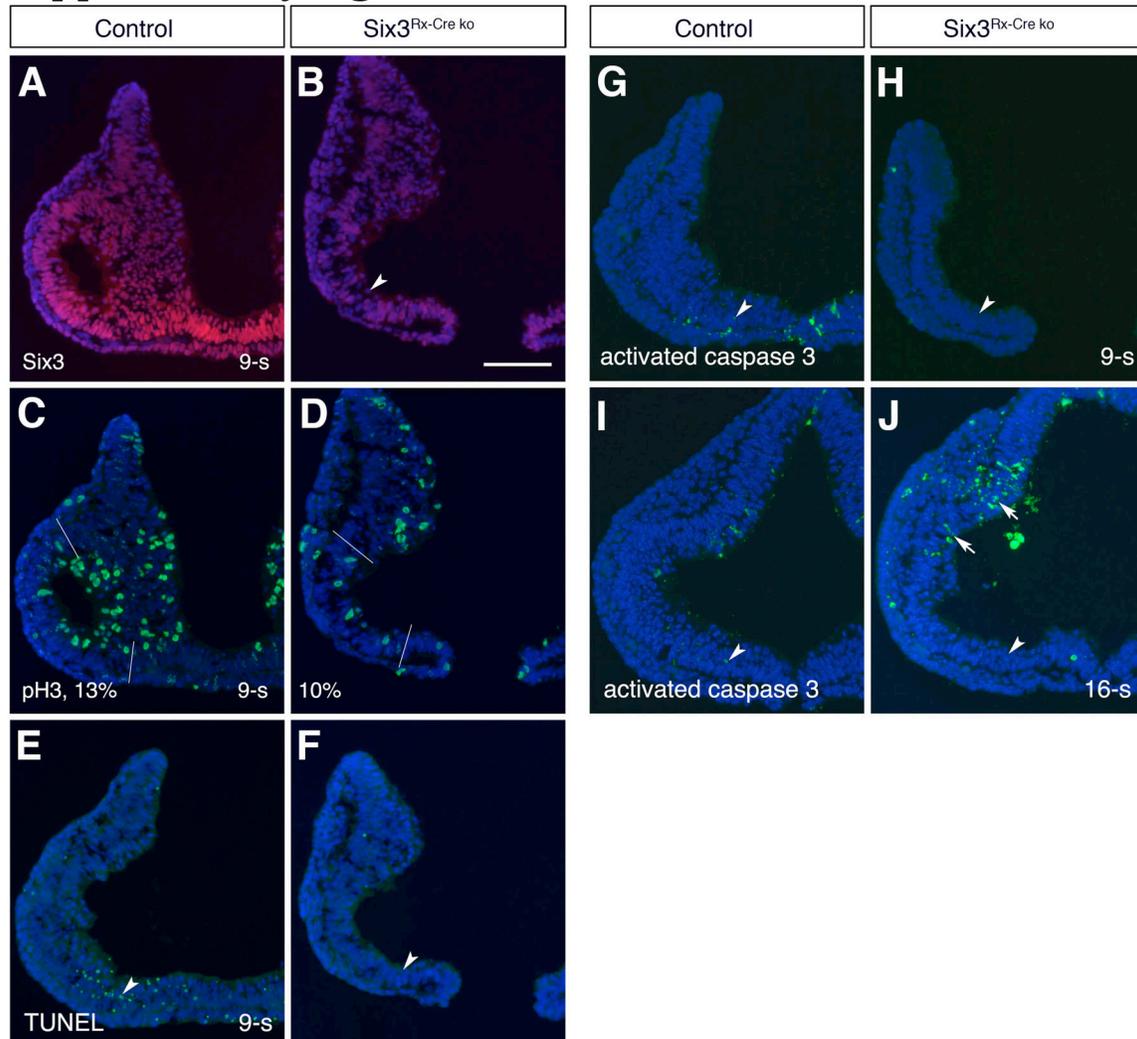
Supp. Figure 1. Rx-Cre is active in the eye field and optic vesicles. To evaluate the temporal and spatial activity of the Rx-Cre transgenic line, we crossed this strain with the R26R reporter mouse line and subjected isolated embryos to X-gal staining. X-gal⁺ cells are detected as early as the head-fold 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 μ m.

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 μ m.

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 μ m.

Supplementary Figure 4

A

			<u>forward</u>	
mouse	000004741	GGGTGGTGGGCAGAGAAAAGCAAGAGAGAGAGGCCACTCCACGCAGTGATTCTGTGGAGG		000004800
	>>>>>>>>			<<<<<<<<<
chick	000000000	-----		000000000
	000004801	GGATTTGCTATGTCCTTTCTCCCCCTCCCTCCTTTGTCCCATCTCCTCGTCCCTTC		000004860
	>>>>>>>>			<<<<<<<<<
	000000000	-----		000000000
	000004861	TGCGTTTGTCCGTCCGGGAGACCCTGGAGTGTCTTTTGATCAGCTCAATCTTCTCTG		000004920
	>>>>>>>>			<<<<<<<<<
	000000001	-----TCTTTTCATCAGTTCATCTCTCTG		000000027
	000004921	GCACTTGAGTTCTGATTGGCTGAAGGACTTAGAGGGAACAA---CTTTAATTAAGTAGA		000004976
	>>>>>>>>			<<<<<<<<<
	000000028	GCACTTGAGTTCTGATTGGCTGAAGGCTTAG-GAAAAAAAAAATCTTTAATTAAGTAGA		000000086
		<u>reverse</u>		
	000004977	TAATCTCTTCTTTAGATGTGAGGTGGCTTCATTTACCA---CCCCTTAACACTG--TTF		000005031
	>>>>>>>>			<<<<<<<<<
	000000087	TAATCTCTTCTTAGGAAGTTCTGCGCTCCATTTACCAACCCCTTTAGCTCTTACTTT		000000146

B

mouse	000026384	TCTACCCCGCCGGAGTCAATGAAACT-TCATTTACATGTTAATCCGCCACTCCGC-C		000026441
	>>>>>>>>			<<<<<<<<<
chick	000013707	CCTCCCTCTGCGGAGTCAATGAAAGTGTCATTTACATGCTAATC--CCCCTCCGCTC		000013764
		<u>forward</u>		
	000026442	CCTTCTCTTCTCACAAACCTTTTCGG-AAAGTGTGGGAAAAGTCTTGGCAGTCAGTA		000026500
	>>>>>>>>			<<<<<<<<<
	000013765	TCT-----TCACAAACCTTTTCGAAAAGTGTGGGAAAAGTCTTGTGGCCCGT		000013817
	000026501	CACAGCTCCCTGCAGC-TCAGACTTCTCCTTGTGTAGTTCAGGGCCCGA-GTGGGGCT		000026558
	>>>>>>>>			<<<<<<<<<
	000013818	TACAGCTCCCTGCCGCTTCGGATTCTTCTGTCTAAGGCCGCGGCCCGTGTGGGTCT		000013877
	000026559	CTCGCTCCAGCAGCGGCTTTTCTCTCCCCCTCCTTTAATTTAAACAAGACTTCAGAGT		000026618
	>>>>>>>>			<<<<<<<<<
	000013878	CTCCCTCCAGCAGG-----CTCATACAAAAAAATCCCAACAAGACTTTGGAGT		000013929
		<u>reverse</u>		
	000026619	TCATCAACCTCCCACTGAAATTCACAGCTGCTGAACAACTAATCCCCTCTCCCGCCTT		000026678
	>>>>>>>>			<<<<<<<<<
	000013930	TCATCAGCTCCCACTGAAATTCACAGCTGCTGAACAACTAATCCCCTCTCTTGGCCTT		000013989
	000026679	TCTTCCCTTCAATGGGCTCTTGGCTTCAAAGACACTTGGGAAAGGACTTTGCTGGGGCT		000026738
	>>>>>>>>			<<<<<<<<<
	000013990	TCTTCCCTTCAATGGGCTCTTGGCTTCAAAGACACTTGGGAAAGGACTTTGTAGGGACT		000014049
	000026739	CACACTGCTTCATCAATAGAAGTTAGCACAAGTATTATCTGAAGCAAGTGGCTTTACA		000026798
	>>>>>>>>			<<<<<<<<<
	000014050	CACACTGCTCTCTCAATAGAAGTTAGTGAAGCATTATCTTCAGCAAGTGGCTTTACA		000014109
	000026799	AACAAATACTGCTTAACAATCCCT-----CCCTCCAAACACACAC--ATCTAGC		000026846
	>>>>>>>>			<<<<<<<<<
	000014110	AACAAATACTGCCTAACAATCTCTGCCCTGCAGCTCCCAACACACACAAACCTAGC		000014169
	000026847	CTGCAGACATGATGGATGGACAACAGATTAGCCTGGTACCCCTGGCTTCACACCCAG		000026906
	>>>>>>>>			<<<<<<<<<
	000014170	CCGCAGACATGTT--ATCTGCAACAGATTAGSAG-----CCCCGTTCTCCATGCCAG		000014220

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.