SUPPLEMENTARY METHODS

Constructs. Chicken *Sox5* coding sequence (Perez-Alcala *et al*, 2004) and a mutant β-catenin where serine 33 is replaced by tyrosine, impeding the phosphorylation necessary for degradation (β-catenin^{CA}, Tetsu & McCormick, 1999), were inserted into pCIG (Niwa *et al*, 1999), that includes an IRES, and EGFP as reporter (pCIG-Sox5, pCIG- β-catenin^{CA}). A human Bcl2-coding sequence inserted into pCDNA3 was used for co-electroporation (Cayuso *et al*, 2006). Four different 22 nt target sequences for cSox5 were chosen to generate short hairpin miRNAs and they were inserted into the pRFPRNAi vector that contains a chicken microRNA operon and dsRFP as reporter gene (pRFPRNAi-Sox5; Das *et al*, 2006). Two of these shRNAs consistently blocked Sox5 expression (mi1, nt 1668-1689; mi2, nt 2049-2070). As a control, a 22nt target sequence based on the luciferase coding sequence was used to generate short hairpin miRNA specific for luciferase (pRFPRNAi-Control; Das *et al*, 2006).

Chick *in ovo* electroporation. Eggs from White-Leghorn chickens were incubated at 38.5ºC in an atmosphere of 70% humidity. Embryos were staged according to Hamburger and Hamilton (HH; Hamburger & Hamilton, 1951).

Chick embryos were electroporated with Quiagen purified plasmid DNA at 1-2 ug/ul in PBS with Fast Green (50 ng/ml), as described previously (Pérez-Alcalá *et al*, 2004). Briefly, plasmid DNA was injected in the lumen of HH10-13 neural tubes, electrodes were placed on either side of the neural tube and a train of electric pulses (5 pulses, 14 volts, 50 msec) was applied using an electroporator (Intracel TSS20). Eggs were further incubated for 24 to 48 hours and they were assayed for EGFP or DsRed expression in the neural tube. The embryos were processed for immunohistochemistry , *in situ* hybridization, western blot or luciferase transcriptional assays.

Immunohistochemistry. Embryos were fixed for 2-4 hours at 4ºC with 4% paraformaldehyde in PBS, and they were immersed in 30% sucrose solution, embedded in OCT and sectioned on a Leica cryostat. Alternatively, embryos were embedded in agarose/sacarose (5%/10%) and they were sectioned in a Leica vibratome (VT1000S). Immunostaining was performed as described previously (Pérez-Alcalá *et al*, 2004).

For BrdU detection, sections were incubated in 2N HCl for 30 minutes and then in 0.1 sodium borate [pH 8.5], before they were exposed to anti-BrdU antibodies. Primary antibodies against the following proteins were used: Sox5 (Pérez-Alcalá *et al*, 2004); green fluorescence protein (GFP; Molecular Probes); phospho-Histone 3 (pH3; Upstate Biochemicals); caspase 3* (BD); HuC/D (Molecular Probes); Brn3a (Chemicon); p27 (Transduction lab). Monoclonal antibodies against BrdU (G3G4), Lhx1/5 (4F2), Pax6, Pax7 and Islet1/2 were all obtained from the Developmental Studies Hybridoma Bank (developed under the auspices of NICHD and maintained by the University of Iowa).

Alexa 488- and Cy3-conjugated anti-mouse or anti-rabbit secondary antibodies (Molecular Probes) were used for detection and after staining, the sections were mounted in Citifluor (Citifluor Ltd., Leicester, UK) plus Bisbenzimide and photograph using a Leica confocal microscope. Cell counting was carried out in 4-8 sections of at least three different embryos from each experimental condition. In the case of BrdU staining, 16-24 optical sections per embryo were counted. The levels of Sox5 protein were measured using the programme Image J [125-175 cells from 5-7 optical sections (25 cells per section) of 3 different embryos for each experimental condition].

S-Phase Labeling and calculation of cell cycle parameters. Cumulative BrdU labeling was carried out by repeated injections of BrdU (5 µg/µl; Sigma) at 2.5 hr

intervals for a total of 1,2, 3, 6, 9, or 12 hr. Calculation was performed by nonlinear regression analysis of BrdU labeling index (percentage of nuclear GFP+ cells that were $BrdU^+$, 1.0 labeling index = 100%) after counting 9-12 confocal sections from at least three embryos for experimental point. Excel spreadsheet was kindly provided by Dr. Richard S. Nowakowski (Nowakowsky *et al*, 1989). In brief, the intercepts of the best nonlinear fit with the abscissa (y) and the time (z) needed to reach the maximum labeling index (growth fraction= GF) correspond to the (1) length of S (TS) relative to the cell cycle (TC) and (2) TC-TS, respectively, allowing us to solve the equations: Tc-Ts = z; (Ts/Tc)GF = y.

For short pulses of BrdU labelling, BrdU was injected into the neural tube 40 minutes prior to fixation.

Fluorescent associated cell sorting (FACS). HH11-13 electroporated neural tubes, carrying GFP as reporter, were dissected out 24-48 hours later and a single cell suspension was obtained after 20 min incubation in Trypsin (Worthington, Lakewood, NJ), followed by a 30 minute fixation in 2% paraformaldehyde as previously described (Morales *et al*, 1998). At least three independent experiments with three separately treated embryos were analysed by FACS for each experimental condition. Dissociated cells were exposed to RNAse A (25 µg/µl) and the nuclei were then labelled with Propidium Iodide (25 µg/µl) to estimate DNA content in GFP^+ cells. Flow cytometry data was collected and a multiparameter analysis was performed in an EPICS XL Coulter Cytometer (Beckman Coulter) with a 488 nm excitation laser, a 525 nm emission filter for GFP and 620 nm emission filter for Propidium Iodide.

***In situ* hybridization.** Embryos were fixed overnight at 4ºC with 4% paraformaldehyde in PBS, rinsed and processed for whole-mount *in situ* hybridization as described previously (Perez-Alcalá *et al*, 2004). The chick *CyclinD1* and *Nmyc* riboprobes have been described elsewhere (Megason and McMahon, 2002; Sawai *et al*, 1990) and *Axin2* cDNA was obtained from the chicken EST project, UK-HGMP RC. Three to seven embryos were analysed for each experimental condition. The probe hybridization was detected with alkaline phosphatase–coupled anti-digoxigenin Fab fragments (Roche). Hybridized embryos were postfixed in 4% paraformaldehyde, vibratome sectioned and immunostained as described above to visualize GFP^+ electroporated cells.

Immunoblot analysis. SDS-8% polyacrylamide gels were calibrated with molecular weight markers (Bio-Rad), and polyclonal anti-SOX5 (Perez-Alcala *et al*, 2004), monoclonal anti-α-tubulin (Promega) and monoclonal β-catenin specific to active form, dephosphorilated on ser37 or Th41(Millipore). Two secondary antibodies (horseradish peroxidase-conjugated, anti-mouse and anti-rabbit) were each used. Bound antibodies were visualized by chemiluminescence using the ECL Advance Western Blotting Detection Kit (Amersham), and luminescent images were obtained by a LuminoImager (AGFA).

Luciferase-reporter assay. Transcriptional activity assays of distinct components of the β-catenin/Tcf pathways were performed in chick embryos electroporated at HH12-13 stage with the indicated DNAs cloned into pCIG or with empty pCIG vector as control, together with a 1 kb of Axin2 promoter in a luciferase reporter construct (Leung *et al*, 2002) or the TOPFLASH containing synthetic Tcf-binding sites (Korinek *et al*, 1998; Upstate) or the same promoters with one or six Tcf-binding sites mutated,

respectively and two *Renilla* luciferase reporter constructs carrying the CMV and the SV40 promoter each (Promega) for normalization. Embryos were harvested after 24 hours incubation *in ovo* and GFP positive neural tubes were dissected and homogenized in Passive Lysis Buffer. Firefly- and renilla-luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega).

METHODS REFERENCES

Das RM et al (2006) A robust system for RNA interference in the chicken using a modified microRNA operon. *Dev Biol* **294**:554-563

Hamburguer V HH (1951) A series of normal stages in the development of the chick embryo. *J Morphol* **88**:49-92

Korinek V, Barker N, Willert K, Molenaar M, Roose J, Wagenaar G, Markman M, Lamers W, Destree O, Clevers H (1998) Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. *Mol Cell Biol* **18**:1248-1256

Morales AV, Hadjiargyrou M, Diaz B, Hernandez-Sanchez C, de Pablo F, de la Rosa EJ (1998) Heat shock proteins in retinal neurogenesis: identification of the PM1 antigen as the chick Hsc70 and its expression in comparison to that of other chaperones. *Eur J Neurosci* **10**:3237-3245

Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**:193-199

Sawai S, Kato K, Wakamatsu Y, Kondoh H (1990) Organization and expression of the chicken N-myc gene. *Mol Cell Biol* **10**:2017-2026

LEGENDS TO SUPPLEMENTARY FIGURES

SFig 1 | Coelectroporation of Bcl2 decrease the levels of apoptosis induced by pCIG and pCIG-Sox5 electroporation. (**A**,**A'**,**D**,**D'**) Sox5 misexpression (pCIG-Sox5; GFP green on right side; **D,D'**) in HH14-16 embryos caused a 260% increase in Cas3* positive dying cells in relation to control pCIG embryos (**A,A'**). (B-C',E-F') Coelectroporation with Bcl2 reduce the number of apoptotic cells 24 and 48 hours postelectroporation. (**I**) Quantification of the effect after 24 or 48 hours post electroporation. (*) $p<0.05$, (**) $p<0.002$, (***) $p<0.001$; n.s. not significant difference.

SFig 2 | Sox5 induces accumulation of cells in G0/G1. (**A-D**) Flow cytometry analysis of the cell cycle phase distribution 24 hours after electroporation with the indicated construct. The DNA content was analysed by Propidium Iodide staining in GFP^+ cells. In control conditions, 45% of cells expressing GFP were in the G0/G1 phase of the cell cycle (2N DNA content), 22% in S-phase (intermediate DNA content) and 34% in G2/M phase (4N DNA content; Fig. 2*L*). Upon Sox5 missexpression there was a 23% increment of cells in G0/G1 (**B**) with respect to the control (**A**)[from 45% to 55% of cells in G0/G1 the ratio of increase is calculated as (55-45)x100/45]. $Sox5^{HIGH}$ expression in embryos with reduced levels of apoptosis (+Bcl2) showed a 38%increase in the rate of cells accumulated in G0/G1 (**D**), respect to the control (**C**).

SFig 3 | Sox5 is necessary to control cell cycle exit. (**A**) A Sox5 specific shRNA (pRFPRNAi-Sox5-mi2) decreased the endogenous levels of Sox5 protein to a 34% in relation to a pRFPRNAi-control 48 hours after electroporation. (**B,C**) Two different Sox5 shRNA (pRFPRNAi-Sox5-mi1 and-mi2) promote a similar reduction in the levels of Sox5 protein in neural progenitors. (**D**) Coelectroporation of Bcl2 decreases the

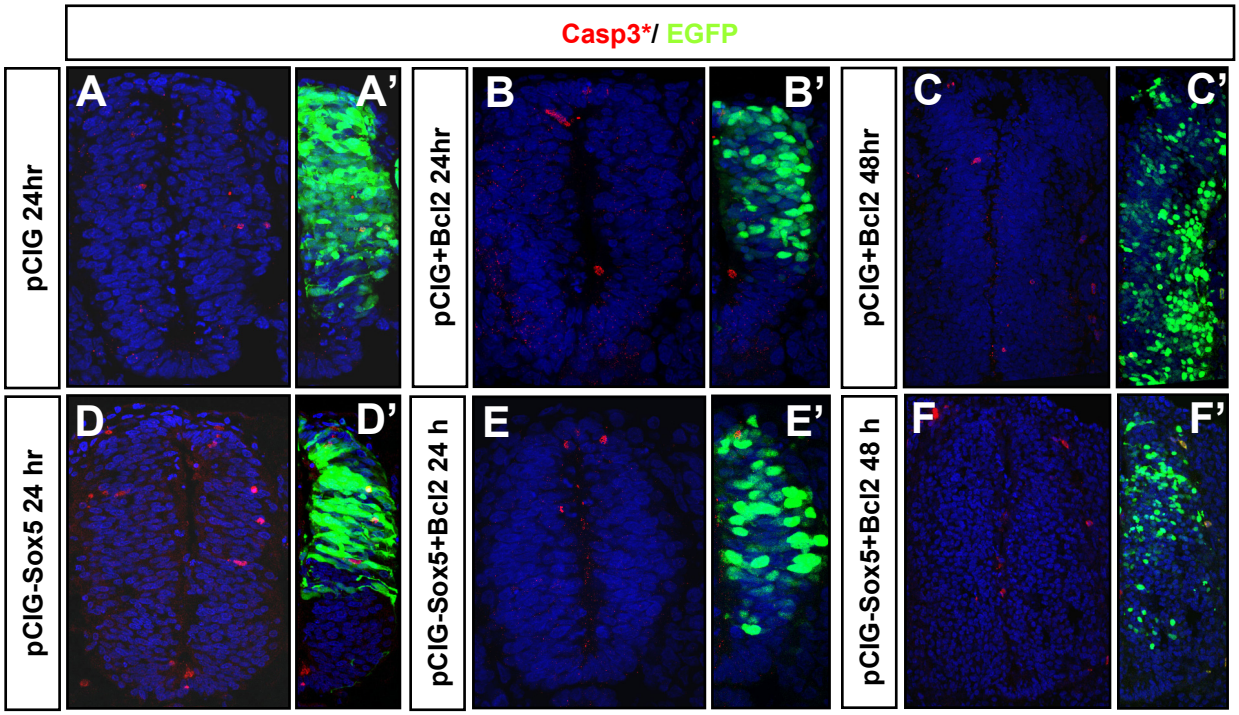
levels of apoptosis induced by pRFPRNAi-Control and pRFPRNAi-Sox5, mi2 electroporation. (**E,F**) Flow cytometry analysis of cell cycle phase distribution of cells electroporated with pRFPRNAi-Control (**E**) or pRFPRNAi-Sox5, mi2 (**F**), in combination with pCIG and the survival factor Bcl2. Knocking down Sox5 levels, in embryos with elevated levels of Bcl2, caused a 51% increase in the ratio of cycling cells in the G2/M phases (**F**), with respect to the control electroporated embryos (**E**). (**)<0.001, (*)<0.05.

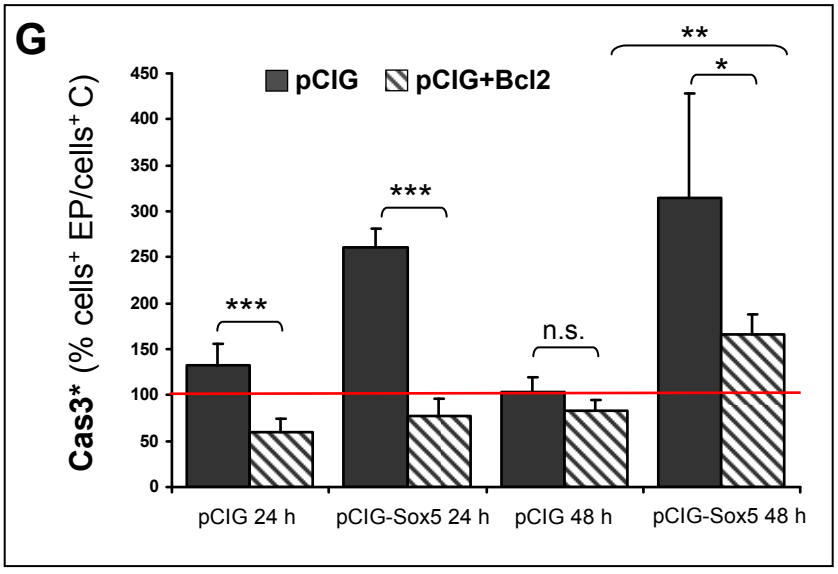
SFig 4 | Sox5 promotes cell cycle arrest by interfering with β-catenin proliferating activity. (**A-D**) Flow cytometry analysis of cell cycle progression in cells transfected with the indicated constructs. pCIG-β-catenin^{CA} promotes a 41% increase in the proportion of neuroepithelial cells in G2/M (**B**) compared with cells transfected with pCIG (**A**). However, elevated levels of Sox5 expression blocked the proliferative effect of β-catenin^{CA} (a 33% decrease in the ratio of cells in G2/M phases in relation to of cells with β-catenin^{CA} alone) (**C**). Conversely, the loss of Sox5 protein in neuroepithelial cells with β-catenin^{CA} potentiates the proliferative effect: a 14% increase in cells in G2/M with respect to cells expressing β-catenin^{CA} alone (**D**) and a 60% respect to pCIG transfected cells (**A**).

SFig 5 | Sox5 interferes with β-catenin transcriptional activity in the spinal cord.(**A**) Quantitative analysis of the transcriptional activity of Sox5 alone or in combination with β-catenin^{CA} on a Tcf (TOPFlash) transcriptional reporter in HH11-3 electroporated neural tubes. Mutations in the six Tcf binding sites (FOPFlash construct) completely abolished the transcriptional activity induced by β-catenin^{CA}, Sox5 or the combination

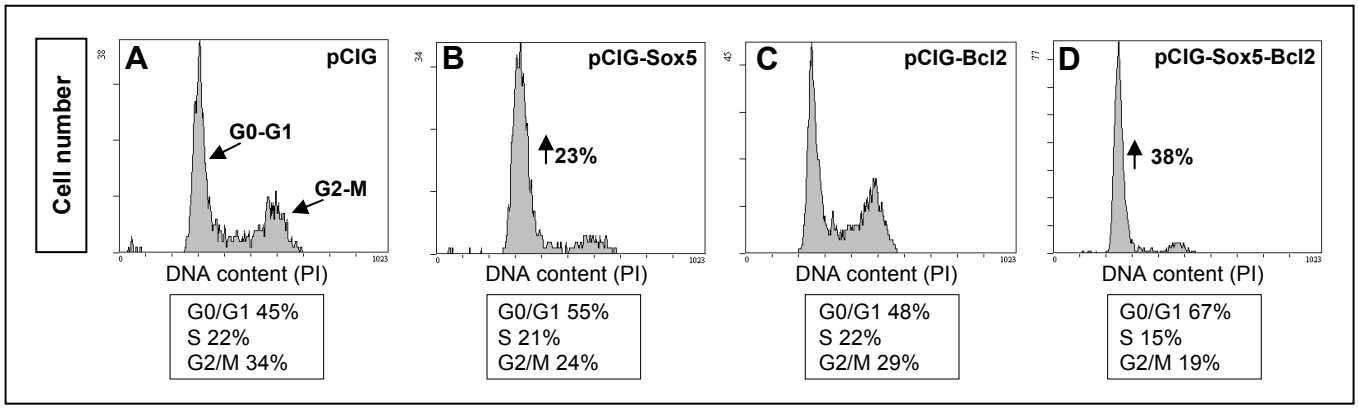
of them both in neuroepithelial cells of the neural tube (data not shown). Graphs show normalized luciferase units relative to the pCIG control.

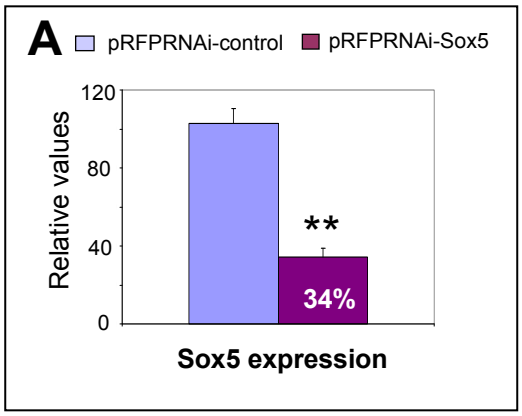
SFig 6 | Preventing apoptosis is not sufficient to rescue the decrease in the number of $Sox5^{HIGH}$ differentiated neurons. (**A-D'**) After 48 hours PE, cells with $Sox5^{HIGH}$ coexpressing the anti-apoptotic protein Bcl2 failed to differentiate as Lhx1/5+ (49% decrease; **C**,**C'**) and Isl1/2+ dI3 (65% decrease; **D,D'**) interneurons with respect to pCIG transfected cells (**A-B'**). (**E**) Quantification of the number of cells expressing a given neural marker 48 hours PE with the indicated construct. (*) $p<0.02$; (**) $p<0.004$; (***) $p<0.001$.

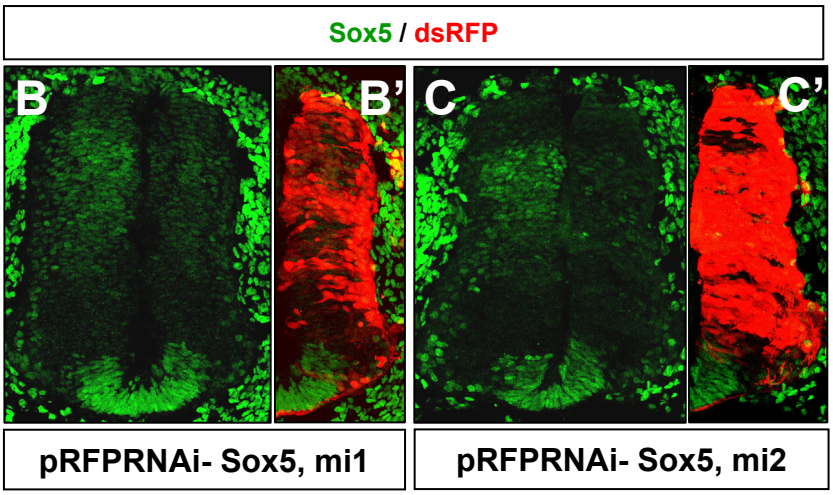

Casp3*/ EGFP
pCIG 24hr
pCIG+Bcl2 24hr
pCIG+Bcl2 48hr
pCIG-Sox5 24 hr
pCIG-Sox5+Bcl2 24 h
pCIG-Sox5+Bcl2 48 h
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'

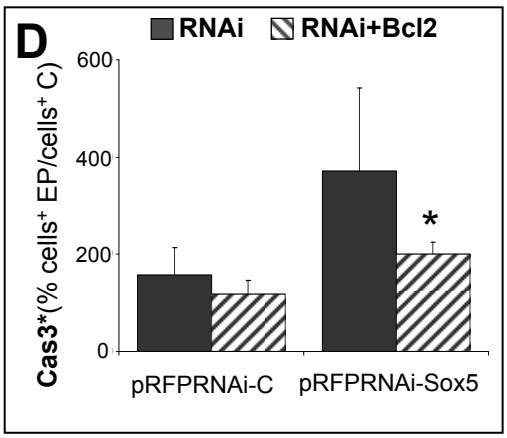

G
pCIG
pCIG+Bcl2
Cas3* (% cells+ EP/cells+ C)
450
400
350
300
250
200
150
100
50
0
***
***
n.s.
**
*
pCIG 24 h
pCIG-Sox5 24 h
pCIG 48 h
pCIG-Sox5 48 h

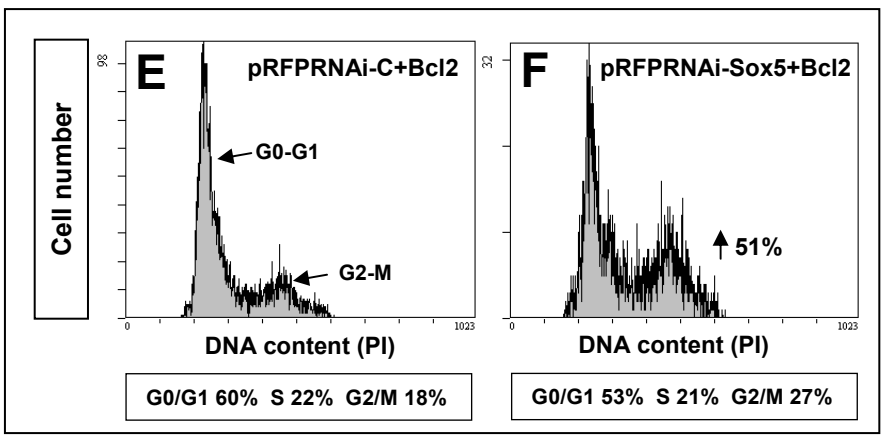
Martínez-Morales_Supplementary Fig 2



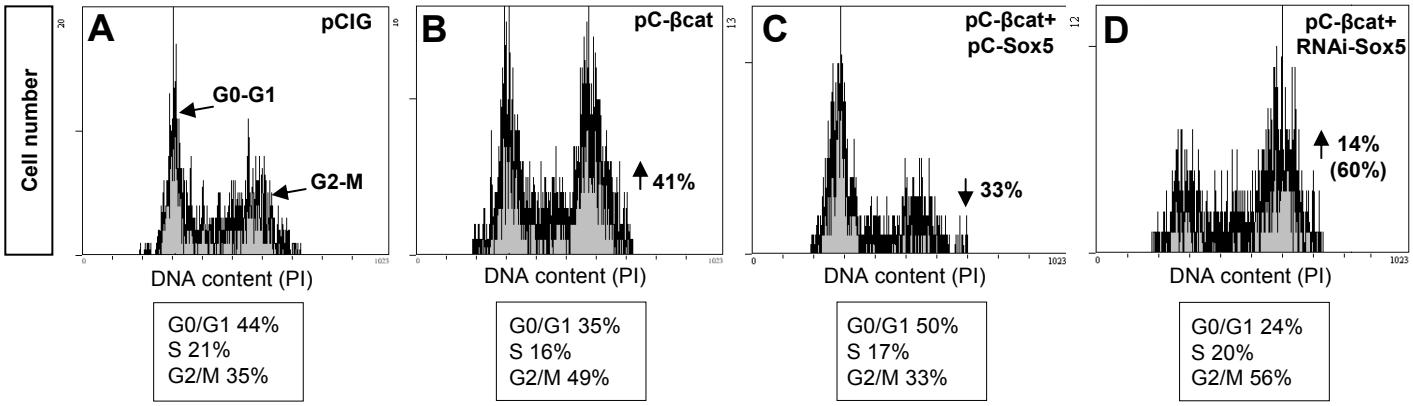

A
pRFPRNAi-control
pRFPRNAi-Sox5
Relative values
120
80
40
0
**
34%
Sox5 expression

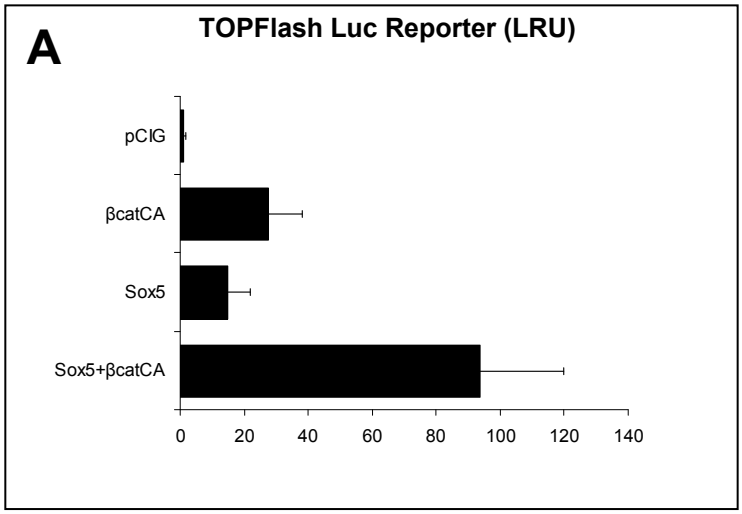

Sox5 / dsRFP
B
B'
C
C'
pRFPRNAi- Sox5, mi1
pRFPRNAi- Sox5, mi2


D
RNAi
RNAi+Bcl2
Cas3*(% cells+ EP/cells+ C)
600
400
200
0
*
pRFPRNAi-C
pRFPRNAi-Sox5


Cell number
E
pRFPRNAi-C+Bcl2
G0-G1
G2-M
DNA content (PI)
G0/G1 60% S 22% G2/M 18%
F
pRFPRNAi-Sox5+Bcl2
51%
DNA content (PI)
G0/G1 53% S 21% G2/M 27%

Martínez-Morales_Supplementary Fig 4




A
TOPFlash Luc Reporter (LRU)
pCIG
βcatCA
Sox5
Sox5+βcatCA
0
20
40
60
80
100
120
140

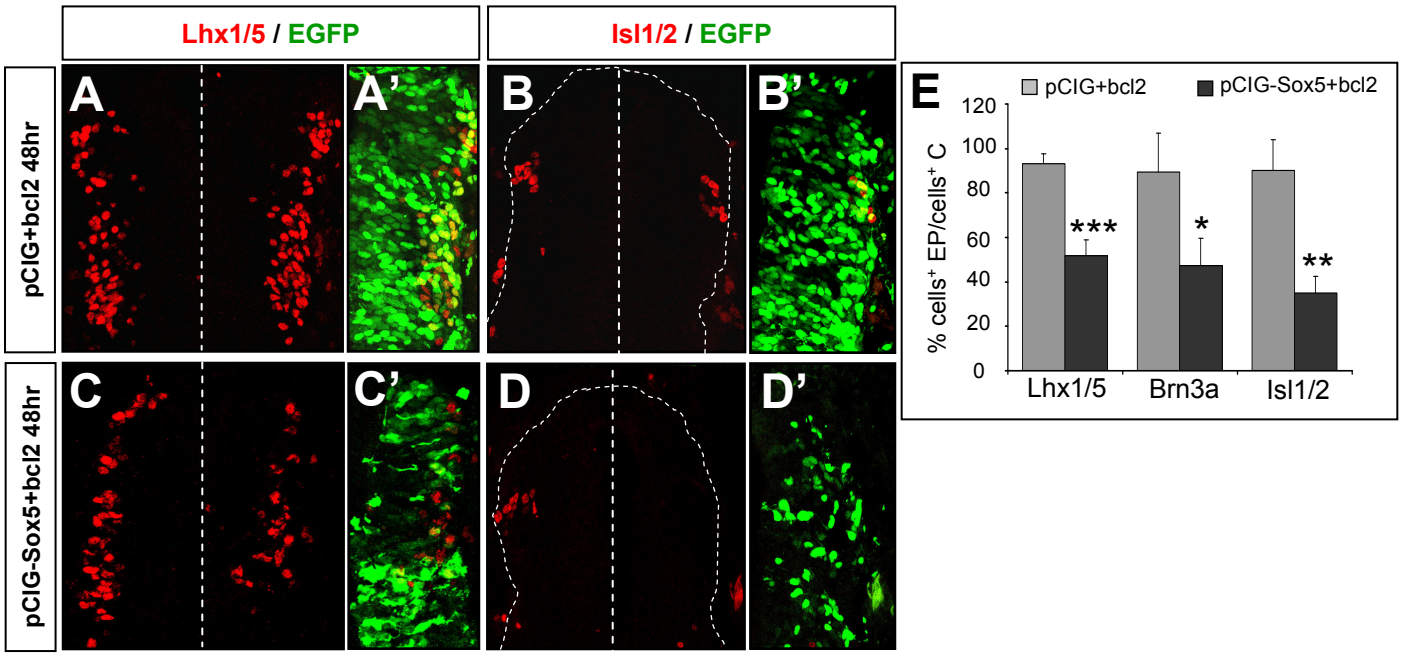

Lhx1/5 / EGFP
Isl1/2 / EGFP
pCIG+bcl2 48hr
pCIG-Sox5+bcl2 48hr
A
A'
B
B'
C
C'
D
D'
E
pCIG+bcl2
pCIG-Sox5+bcl2
% cells+ EP/cells+ C
120
100
80
60
40
20
0
***
*
**
Lhx1/5
Brn3a
Isl1/2

Table S1. Analysis of hemitube thickness and cell density(cells/ μm²) in HH14-17 chick embryos electroporated with pCIG or pCIG-Sox5.

Construct	Hemitube thickness (μm)			Cells/ 100 μm²		
	EP	No EP	% EP/No EP	EP	No EP	% EP/No EP
pCIG	59.2±7.2	57.4±6.9	103.1±0.1(n.s)	1.54±0.01	1.57±0.01	98.12±0.18 (n.s)
pCIG-Sox5	41.8±4.9	60.5±4.8	**69.4±3.3 (*)**	1.75±0.31	1.66±0.24	104.86±4.95 (n.s.)

(n.s.) not signifcant differences; (*) p<0.001