Sox5 controls cell cycle progression in neural progenitors by interfering with Wnt/β-catenin pathway

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Genes of the *Sox* family of HMG-transcription factors are essential during nervous system development. Here, we show that Sox5 is expressed by neural progenitors in the chick spinal cord and is turned off as differentiation proceeds. Over-expression of Sox5 in neural progenitors causes premature cell cycle exit and prevents terminal differentiation. Conversely, knocking down Sox5 protein extends the proliferative period of neural progenitors and causes dramatic cell death in a dorsal interneuron (dI3) population. Moreover, Sox5 reduces Wnt/β-catenin signalling triggering the expression of the negative regulator of the pathway Axin2. We propose that Sox5 regulates the timing of cell cycle exit by opposing Wnt/β-catenin activity on cell cycle progression.

INTRODUCTION

During the development of the central nervous system (CNS), a large number of different neurons and glial cells are generated from a small population of self-renewing stem and progenitor cells. In the vertebrate spinal cord mitotically active and postmitotic cell populations are spatially segregated. Thus, neural progenitors are located in the medial ventricular zone and they migrate laterally to the mantle zone upon exiting the cell cycle, a site where differentiating cells accumulate.

A dorsal-ventral gradient of the Wnt /β-catenin/Tcf pathway positively regulates cell cycle progression of spinal neural progenitors through CyclinD1, CyclinD2 and Nmyc (Megason & McMahon, 2002).

However, it is still not clear how the progression of the proliferation programme, promoted by signals like Wnt, can be counteracted to facilitate the initiation of the

neurogenic programme. The HMG-box transcription factors of the *Sox* gene family could be at the core of some of those processes, as they have essential regulatory functions during neurogenesis in the CNS (Wegner & Stolt, 2005). In the spinal cord, Sox1-3 proteins (SoxB1 group) preserve cells in an undifferentiated state (Bylund *et al*, 2003). By contrast, SoxB2 factors promote the initiation of the differentiation programme (Sandberg *et al*, 2005). Sox5 belongs to the SoxD group and it is involved in the formation of the cephalic neural crest (Perez-Alcala *et al*, 2004), as well as in the control of the cell fate of distinct corticofugal neurons (Lai *et al*, 2008).

Here, we show that Sox5 is expressed in neural progenitors in the spinal cord, and in dorsal dI3 interneurons. Through gain- and loss-of-function analyses, we found that Sox5 controls the timing of cell cycle exit by neural progenitors at the G1-S transition by counteracting the mitotic effect of the Wnt/β-catenin pathway. We provide evidence to suggest that Sox5 does this by controlling the feedback repressor pathway regulating Wnt signalling. Furthermore, we have found that Sox5 downregulation in postmitotic cells is necessary for the progression of the differentiation program. Hence, these data situate Sox5 as an important brake of Wnt/β-catenin mitogenic activity during the progression of neurogenesis.

RESULTS AND DISCUSSION

Sox5 is expressed in neural progenitors in the spinal cord and in a subset of interneurons and motoneurons

In order to determine the possible role of Sox5 in early neurogenesis, we defined the pattern of Sox5 expression at the midtrunk level of the spinal cord in chicken embryos at stages 10-24 of Hamburguer and Hamilton (HH10-24). At HH10, when most of the neuroepithelial cells are proliferating progenitors, very low levels of Sox5 were detected

in these progenitors whereas higher levels were observed in the dorsal premigratory neural crest cells (Fig 1A). By HH14, when around 12% of the neural progenitors have exited the cell cycle and have started differentiation at the marginal zone (Wilcock *et al*, 2007), Sox5 expression increased in progenitors (Fig 1B). Finally, from HH14 to HH24, when neural differentiation is more active and gliogenesis has not yet started, Sox5 expression disappeared from most of the interneurons that expressed the pan-neural marker HuC/D (Fig 1C,D). At HH24, Sox5 expression remained in neural progenitors expressing Pax7 (Fig 1E,F). Dorsally, only Islet1/2$^+$ dorsal interneurons (dI3) expressed Sox5 (Fig 1G-I). Ventrally, Sox5 was expressed by a small subpopulation of the Islet1/2$^+$ motoneurons (Fig 1J-L). This dynamic pattern of expression suggests a possible role for Sox5 in the control of the transition from proliferation to differentiation.

Sox5 controls the timing of cell cycle exit

To explore the function of Sox5 in neurogenesis, we electroporated a pCAGGS-Sox5-IRES-GFP vector (pCIG-Sox5) and prematurely increased Sox5 levels (Sox5^{HIGH}) in neural progenitors at stage HH10-13. Sox5^{HIGH} provoked a 30.6±3.3 % decrease in the size of the electroporated right hemi-tube 24 hours post electroporation (PE; stage HH14-17) when compared to the left control side (Fig 2E; supplementary Table1) or with an electroporated control neural tube (pCIG; Fig 2A). There were not significant changes in cell density in Sox5 electroporated neural tubes (supplementary Table1). The change in the hemitube size was due, in part, to a substantial decrease in proliferation observed by a reduction in BrdU incorporation after a 40 min pulse (only 61% ± 11 incorporated BrdU; Fig 2 F,B,I) and in the number of cells in M phase expressing phospho-Histone H3 (PH3; only 73±13%; Fig 2G,C,I) in comparison with control

embryos. This reduction was even more dramatic 48 hours PE (at HH19-23) when the proportion of PH3$^+$ cells decreased to 46±7% (Fig 2I) and that of BrdU$^+$ cells fell to 59±9% (Fig 2I). The reduction in proliferation in Sox5^{HIGH} cells was accompanied by an increase in apoptosis 24 hours (260±20%; Fig 2I and supplementary Fig 1D) and 48 hours PE (314±114%; Fig 2I) in relation to control cells (Fig 2I and supplementary Fig 1A). Consequently, a premature increase in Sox5 reduces the total number of neuroepithelial cells due to both a reduction in cell proliferation and activation of apoptosis.

To further explore if the neural cells with Sox5^{HIGH} had an altered cell cycle phenotype and not only an increased death rate, we analysed the cell cycle distribution of surviving cells by flow cytometry. After 24 h of Sox5 electroporation, the ratio of neuroepithelial cells accumulated in G0/G1 phases was increased by 23% with respect to control electroporated cells in G0/G1 (a 10% increase respect to the total population; supplementary Fig 2A,B). In accordance, the expression of *CyclinD1* and *Nmyc* were severely reduced in neural progenitors (Fig 3H; data not shown).

To clarify if neural progenitors overexpressing Sox5 were exiting the cell cycle (accumulated in G0) or retained in a longer G1 phase due to the reduction in *CyclinD1* levels (Lange *et al* 2009), cumulative BrdU labelling was performed (Nowakowski *et al*, 1989; Fig 2J). We found that Sox5^{HIGH} neural progenitors had a similar cell cycle length to pCIG control progenitors (14.8 and 14.4 hr, respectively) and similar S phase duration (4.5 and 4.7 hr respectively). However, there was a 12% decrease in the proportion of Sox5^{HIGH} cycling cells respect to the control (growth fraction of 0.75±0.03 versus 0.85±0.05; Fig 2J). In conclusion, Sox5 promotes premature cell cycle exit in neural progenitors without significantly affecting cell cycle length.

As for the increase in apoptosis, we have found that while 53±8% of the pCIG apoptotic cells were BrdU$^+$ progenitors (after a 2 hr pulse), only a 28±6% of the Sox5^{HIGH} apoptotic cells were BrdU$^+$. Additionally, there were no changes in the number of apoptotic cells that expressed the differentiation marker HuC/D (15±6% for the pCIG and 13±4% for the Sox^{HIGH} apoptotic cells; data not shown). Moreover, using the Bcl2 survival factor to rescue this cell death (Cayuso *et al*, 2006; supplementary Fig 1B-G), we observed that neuroepithelial cells with high levels of Sox5+Bcl2 preferentially accumulated at G0/G1 (38% increase; supplementary Fig 2C,D). Thus, apoptosis caused by Sox5 overexpression predominantly occurs in postmitotic neural cells before they acquire definitive neuronal markers.

We next addressed whether Sox5 was not only sufficient but was also necessary to control the balance of cell proliferation-cell cycle exit. Knocking down Sox5 expression by specific interfering short hairpin RNAs (pRFPRNAi-Sox5) caused a dramatic 66±4% reduction in Sox5 protein levels 48 hours PE at HH19-22 (Fig 3A-B; supplementary Fig 3A). Neural progenitors with reduced Sox5 levels, upon using two different interferent RNAs (mi1 and mi2), presented a higher frequency of both BrdU incorporation (up to 114±7%; Fig 3D,C,G) and PH3 staining (up to 130±19%; Fig 3 E-G). Moreover, a small number of cells transfected with RNAi-Sox5 and expressing PH3 were located in the mantle layer (insets in Fig 3F), suggesting that they entered mitosis in an ectopic position. In fact, reducing Sox5 levels increased *CyclinD1* expression (n=3; Fig 3I). That could account for the appearance of ectopic mitosis as long-term forced expression of *CyclinD1* leads to the appearance of proliferating cells in the differentiating field (Lobjois *et al*, 2008).

With respect to apoptosis, neural progenitors with low Sox5 levels presented up to a 2.4-fold increase in cell death when assessed by Cas3* expression and by the

visualization of pyknotic nuclei (Fig 3G). It is possible that cells forced to proliferate when Sox5 expression is compromised initiate apoptosis while going through the G2/M phases. In neural tubes coexpressing Bcl2 and pRFPRNAi-Sox5, we observed a 51% increase in the ratio of cells accumulated in G2/M with respect to control neural tubes by flow cytometry analysis (supplementary Fig 3C,D). Altogether, this suggests that neural progenitors with reduced Sox5 expression are maintained for longer in a proliferative state and a fraction of them die by apoptosis.

In summary, Sox5 negatively regulates cell cycle progression and it is necessary and sufficient to promote cell cycle arrest at the G1-S transition.

Sox5 induces cell cycle exit by interfering with β-catenin transcriptional activity

The Wnt/β-catenin signalling pathway favours neural tube progenitor proliferation directly controlling the transcription of the cell cycle regulators *CyclinD1* and *Nmyc* (Tetsu & McCormick, 1999; ten Berge *et al*, 2008; Fig 4A-B).

To test whether Sox5 could control cell cycle exit by interfering with the Wnt/β-catenin pathway, we electroporated Sox5 together with a more stable form of β-catenin (β-catenin^{CA}; Tetsu & McCormick, 1999), lacking one of the four phosphorylation sites that mediate Axin/APC complex binding and degradation. As expected, neuroepithelial cells with β-catenin^{CA} displayed a higher proportion of cells in G2/M with respect to the control (supplementary Fig 4A,B). The expression of Sox5^{HIGH} reverted this situation, causing a 33% reduction in the ratio of progenitors in G2/M phases with respect to cells expressing β-catenin^{CA} alone (supplementary Fig 4B,C). Moreover, the increase in *CyclinD1* and *Nmyc* expression mediated by β-catenin^{CA} was prevented in a cell autonomous manner (n=3; asterisk versus arrow in Fig 4E). Additionally, *CyclinD1* was

expressed in adjacent non electroporated cells probably by the induction of soluble signals coming from the cells electroporated with Sox5.

Conversely, knocking down Sox5 expression provoked a further 14% increase in the ratio of cells in the G2/M phase with respect to cells expressing β-catenin^{CA} alone (supplementary Fig 4D). Thus, in neural progenitors with reduced Sox5 levels, the proliferative potential of the Wnt pathway is reinforced.

Next, we determined that Sox5-induced changes in gene expression (Fig 4A-H) were due to alterations in the Wnt canonical pathway activity as we observed a 51±15% decrease in the levels of the dephosphorylated active form of β-catenin (Fig 4I) in Sox5^{HIGH} cells with respect to control cells. Surprisingly, using the TOPFLASH reporter of Wnt/β-catenin transcriptional activity, in neural tube cells, we observed that Sox5 acted synergistically with β-catenin^{CA} increasing Tcf reporter activity by 3.4 folds respect to β-catenin^{CA} alone (supplementary Fig 5).

In order to conciliate these apparently opposing results, we analysed the transcription of negative regulators of the Wnt pathway such *Axin2*, (Leung *et al*, 2002). Using the luciferase reporter system under the control of 1kb of the *Axin2* promoter (with a functional Tcf binding site; Leung *et al*, 2002), we found that Sox5 acted synergistically with β-catenin^{CA} increasing *Axin2* promoter activity by 3.3 folds respect to β-catenin^{CA} alone (Fig 4J). In fact, this Sox5 function is dependent on the Tcf binding site in the *Axin2* promoter (Fig 4J). Furthermore, *Axin2* was overexpressed in the dorsal progenitors expressing Sox5^{HIGH} alone (Fig 4H) and/or with β-catenin^{CA} (Fig 4G,C) in relation to control (Fig 4D). In conclusion, Sox5 enhances Tcf/β-catenin activity on the transcription of *Axin2* in neural progenitors.

The increased levels of Axin2 could mediate the reduction in the active form of β-catenin observed in these cells and consequently downregulate expression of *CyclinD1*

and *Nmyc,* leading to cell cycle exit (Fig 4K). Obviously, *Axin2* transcription would also be affected by the reduction in the active form of β-catenin. A plausible interpretation for the elevated levels of *Axin2* mRNA would be that Sox5 could be enhancing/stabilizing Tcf /β-catenin transcriptional activity preferentially in the context of the *Axin2* promoter, compensating for the reduction in active β-catenin. There are examples of *Sox* genes, such as *Sox4*, that enhances Tcf/β-catenin activity and may function to stabilize β-catenin (Sinner *et al*, 2007). We can not exclude that in the context of *CyclinD1* or *Nmyc* promoter Sox5 could reduce the Tcf/ β-catenin activity, as the same Sox protein can exert a different transcriptional modulation in distinct genes and also depending on a given developmental scenario (Wegner & Stolt, 2005).

Downregulation of Sox5 expression is required for the progression of dorsal interneuron differentiation

Proliferation and differentiation are highly coordinated events that can be uncoupled on occasion. To determine whether this was the case following Sox5 premature expression, specific markers of dorsal and ventral interneurons were analysed (Jessell, 2000). Neural progenitors with Sox5[HIGH] generated neurons that prematurely expressed Lhx1/5 (dI2,4,6 dorsal and V0,2 ventral interneurons; Martí *et al*, 2006), before reaching the mantle zone (n=4/8; Fig 5B). Moreover, they ectopically expressed the cell cycle inhibitor p27[kip1+] (n=3; Fig 5C-D). However, there was a drastic reduction in Lhx1/5[+] interneurons located in the mantle zone 24 hours (n= 8/8; Fig 5B) and 48 hours after maintaining Sox5[HIGH] (54±5% remained; n= 4; Fig 5E,F,K). A similar reduction was observed for Brn3a^{+} interneurons (dI3,5; 49±10%; n=3; Fig 5K), and for the En-1^{+} V1 interneurons (38±19%; n=4; Fig 5K). More dramatically, only 13±4% of the Isl1/2[+] interneurons (dI3) remained (n=3; Fig 5K). Reducing apoptosis with the anti-apoptotic

protein Bcl2 did not significantly recover the reduced populations of Lhx1/5[+] and Brn3a^{+} interneurons, and only partially rescued that of Isl1/2[+] interneurons (35±7% remained; supplementary Fig 6A-E). In conclusion, progenitors with Sox5[HIGH] were forced to exit the cell cycle, generated neurons that prematurely expressed differentiation markers and around half of neurons fail to complete the differentiation program.

On the other hand, it has been shown that forcing progenitors to continue to cycle does not prevent cells from differentiating into the right cell type (Dyer & Cepko, 2000; Lobjois *et al*, 2008). In fact, knocking down Sox5 levels does not alter the hemitube thickness and only mildly alters the gross pattern of differentiation, as it slightly reduced the number of Lhx1/5[+] interneurons (16% reduction; n=5; Fig 5G,L) and reduced the number of the small population of Isl1/2[+] dI3 interneurons (by 82%; n=3; Fig 5 I,L). The reduction in the number of Lhx1/5[+] and Isl1/2^{+} neurons was totally rescued by Bcl2 protein (Fig 5H,J,L). As the apoptosis is not fully overcome by Bcl2 expression (supplementary Fig 3D), these results would suggest that progenitors with reduced levels of Sox5 remain cycling for longer and probably generate an increased number of Lhx1/5[+] differentiated interneurons, similar to CyclinD1-transfected neural progenitors (Lobjois *et al*, 2008). The possible excess of Lhx1/5[+] interneurons would have been overcompensated by apoptosis.

In summary, these results suggest that: i) Sox5 is sufficient to induce premature cell cycle exit but it prevents the progression of the interneurons differentiation program; ii) Sox5 is required for the timing of cell cycle exit and for the correct final number of dorsal interneurons and iii) Sox5 is essential for the survival of dI3 interneurons, that normally express high level of Sox5.

Our data establish, for the first time in a neural context, both the role of a Sox transcription factor in the timing of cell cycle exit and in the modulation of the Wnt/β-catenin pathway to control that function. By increasing the levels of the negative regulator *Axin2*, Sox5 would control the feedback repressor pathway regulating Wnt signalling (Leung *et al*, 2002).

Several Sox proteins fulfil crucial roles in the context of neurogenesis. SoxB1 promote progenitor cell maintenance (Bylund *et al*, 2003) while SoxB2 promote the onset of neuronal differentiation (Sandberg *et al*, 2005). Our studies assign a role for Sox5 between the activity of SoxB1 and SoxB2 proteins, since Sox5 promotes cell cycle exit of neural progenitors and its downregulation is required for the progression of neuronal differentiation.

METHODS

Chick *in ovo* electroporation. Embryos were electroporated at HH10-13 and processed 24 hours PE (HH14-17) or 48 hours PE (HH19-22) as previously described (Perez-Alcala *et al*, 2004), using immunohistochemistry, *in situ* hybridization, western blot, FACS or luciferase assays.

Fluorescent associated cell sorting (FACS). Electroporated neural tubes, carrying GFP as reporter, were dissociated into single cell suspension 24-48 hours later as previously described (Cayuso *et al*, 2006). Nuclei were labelled with Propidium Iodide to estimate DNA content in GFP$^+$ cells. Flow cytometry data was collected and multiparameter analysis was performed in an EPICS XL Coulter Cytometer (Beckman Coulter).

Luciferase reporter assay. Transcriptional activity assays were performed in embryos electroporated with the indicated DNAs, 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) and two *Renilla* luciferase reporter constructs carrying the CMV and the SV40 promoter each (Promega) for normalization. Luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega).

Statistical analysis. All data presented here are number of cells in the electroporated area that expresses a marker respect to the cells expressing the same that marker in an equivalent area in the non electroporated side (% cells$^+$ EP/cells$^+$ C). Quantitative data were expressed as mean ±s.d. or SEM.; n≥3 embryos per experimental point. Significant differences were tested by Student's test.

Supplementary information is available at *EMBO reports* online.

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LEGENDS TO FIGURES

Fig 1 | Expression of Sox5 in the developing chick spinal cord. (**A**) At stage HH10, Sox5 is expressed dorsally in neural crest cells and in floor plate cells. (**B**) At stage HH14, Sox5 is expressed by the majority of the neuroepithelial cells. (**C,D**) Sox5 expression at HH24 is absent from most of the HuC/D differentiating interneurons. (**E, F**) Sox5 is co-expressed in dorsal neural progenitors with Pax7. (**G-I**) A subpopulation

of dorsal dI3 interneurons co-expresses Sox5 and Islet1/2 (arrows). (**J-L**) At HH24, Sox5 is expressed in a subpopulation of Isl1/2$^+$ motoneurons (arrows).

Fig 2 | Forced Sox5 expression promotes cell cycle exit. Sox5 misexpression (pCIG-Sox5; GFP green on right side; **E-H'**) in HH14-16 embryos caused a 30% reduction in the size of the hemitube (**E**), in the number of BrdU (**F,F'**) and pH3 positive cells (**G, G'**) and an increase in Cas3* positive dying cells (**I**) in relation to control pCIG embryos (**A-C'**). Expression of the neural progenitors maker Pax6 is not altered in Sox5^{HIGH} cells (**H, H', D,D'**). (**I**) Quantification of the effect 24 or 48 hours PE. (*) $p<0.01$; (**) $p<0.005$; (***) $p<0.001$. (**H**) Cumulative BrdU labelling curves of pCIG (black squares) or pCIG-Sox5 (red circles) electroporated neural tube cells. Dashed lines indicate the reduction in growth fractions. Mean of three embryos per experimental point, s.d. and t test was calculated; (***) $p<0.01$; (**) $p<0.025$; (*) $p<0.05$
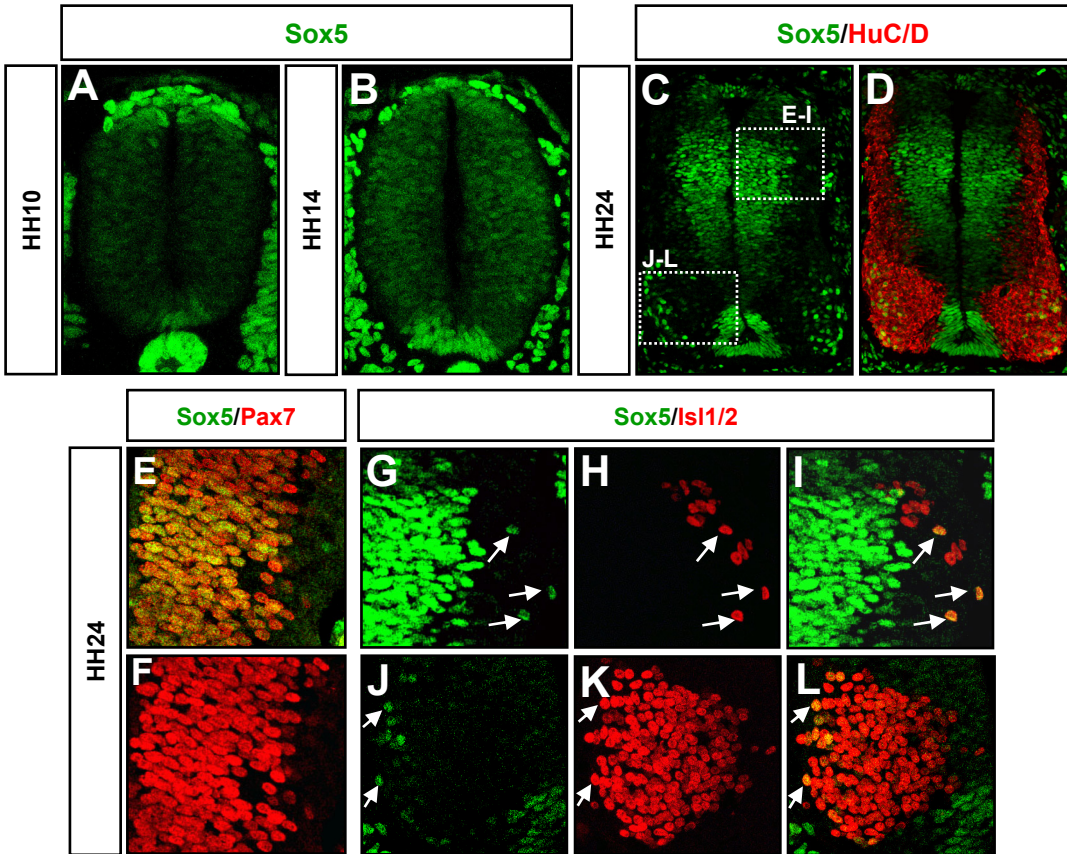
Fig 3 | Sox5 is necessary to control the timing of cell cycle exit. (**A-G**) Stage HH22, embryos analysed 48 hours PE. (**A-B'**) Sox5 specific shRNA (pRFPRNAi-Sox5, mi2) decreases a 66% the endogenous levels of Sox5 protein (**B,B'**) in relation to a pRFPRNAi-control (**A,A'**). (**C-G'**) Knocking down Sox5 expression with mi2 increases the number of BrdU (**D,D'**) and PH3 (**F, F'**) positive cells respect to control (**C,C',E,E'**). Few proliferating RFP/PH3 double positive cells are misallocated in the differentiated cell area (arrows in F, inset in **F'**). (**G**) Quantification of the effects using two different shRNAs, mi1 and mi2. (*) $p<0.05$; (**) $p<0.025$; (***) $p<0.01$; n.s. not significant. (**H,-I'**) Sox5 overexpression decreases *CyclinD1* expression at HH14
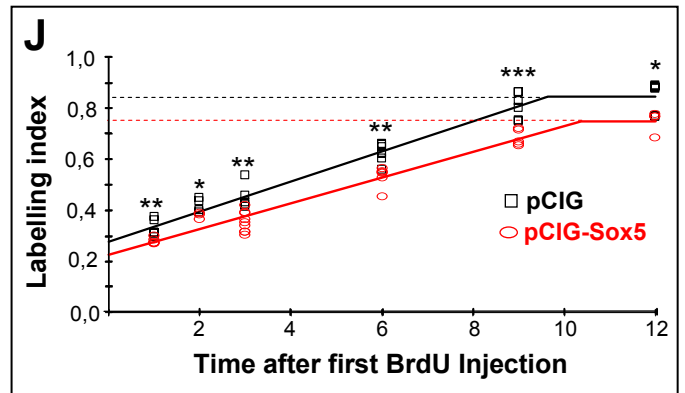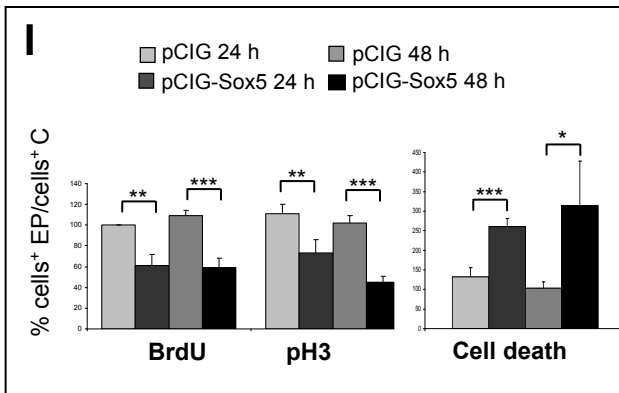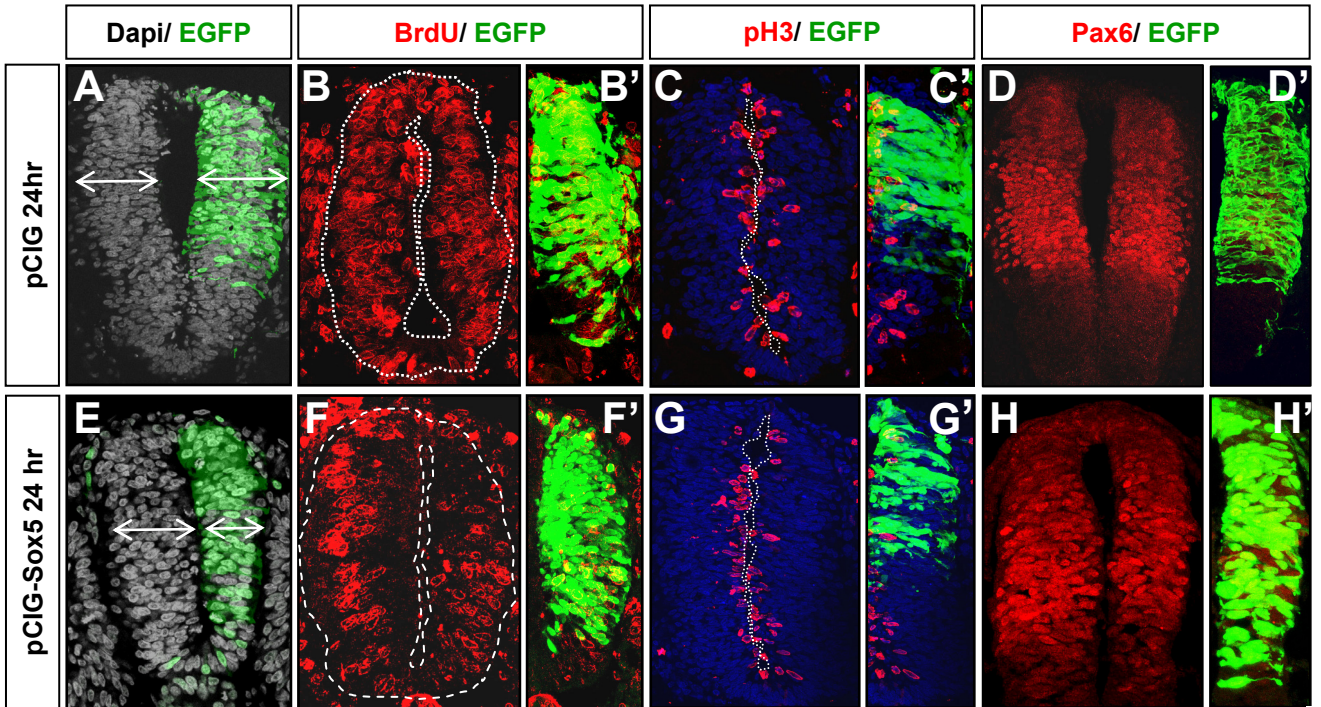
(asterisk in **H**) while reduction of Sox5 levels induces *CyclinD1* expression at HH18 (arrow in **I,I′**).
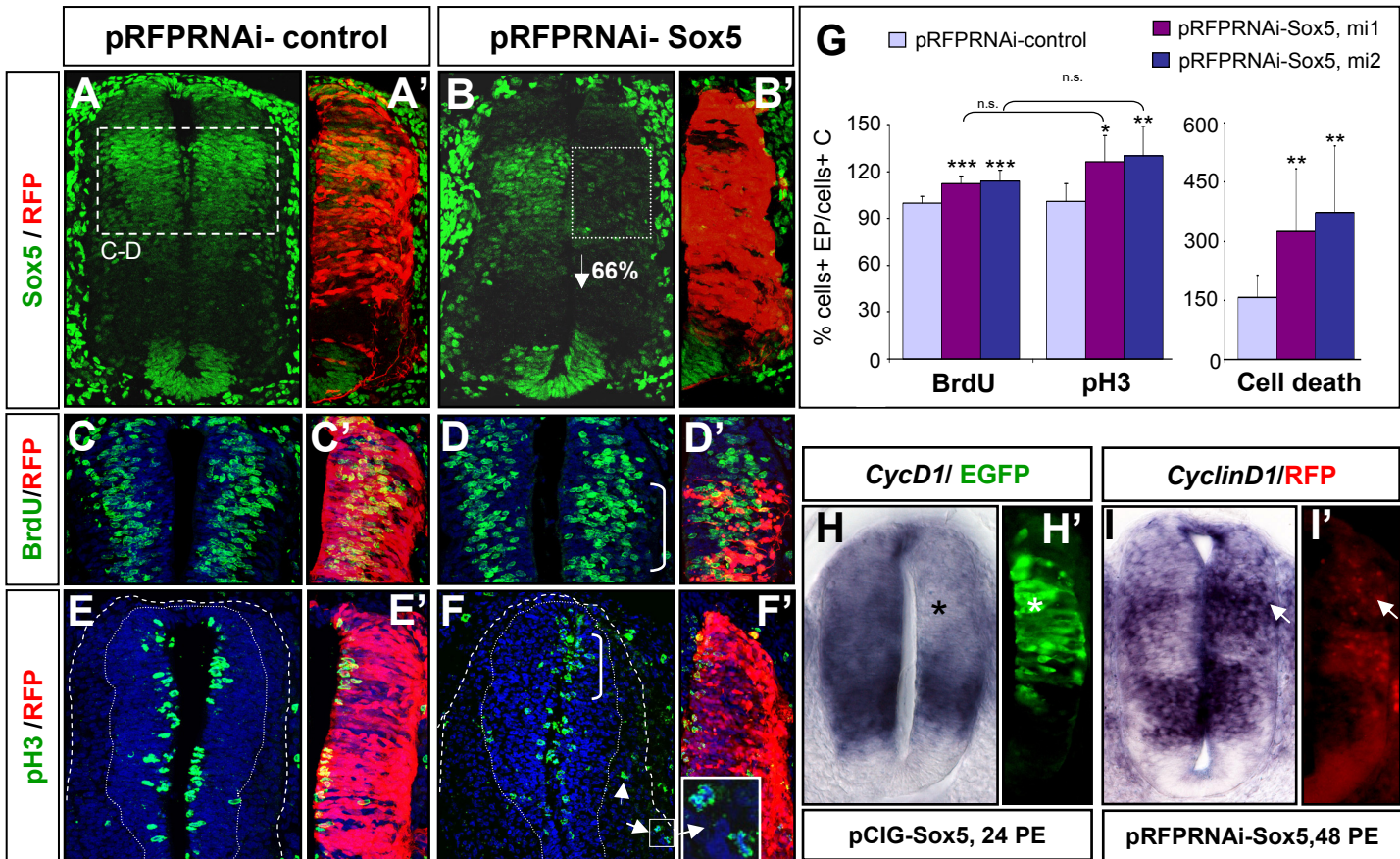
Fig 4 |Sox5 interferes with β-catenin transcriptional activity in the control of *Axin2* expression. (**A-J**) Stage HH14-16 embryos analysed 24 hours post electroporation. Activation of the canonical Wnt pathway by overexpressing a stabilized form of β-catenin (pCIG-β-catenin^{CA}) promotes an upregulation of *CyclinD1* (arrow in **A,A'**), *NMyc* (**B,B'**) and *Axin2* expression (**C,C'**). Forcing Sox5 expression together with pCIG-β-catenin^{CA} represses *CyclinD1* (asterisk versus arrow in **E,E'**) and *NMyc* (**F,F'**) expression and induces *Axin2* expression (**G,G'**). Sox5^{HIGH} alone also induces *Axin2* expression (**D,D'** compared to H,H'**)** . (**I**) Sox5 overexpression reduces to a 49% the levels of active β-catenin (β-cat*) relative to those in pCIG electroporated cells. Values were normalized using αtubulin as a reference (αtub). (*) $p<0.005$. (**J**) Quantitative analysis of the transcriptional activity of Sox5 alone or in combination with β-catenin^{CA} on an intact Axin2 promoter (light bars) or on a Tcf binding site mutated one (dark bars). Graphs show normalized luciferase units relative to the pCIG control. Each bar represents mean ± SEM of triplicate experiments. (**) $p<0.001$. (**K**) Model for Sox5 action on Wnt signalling pathway in the spinal cord (see main text).
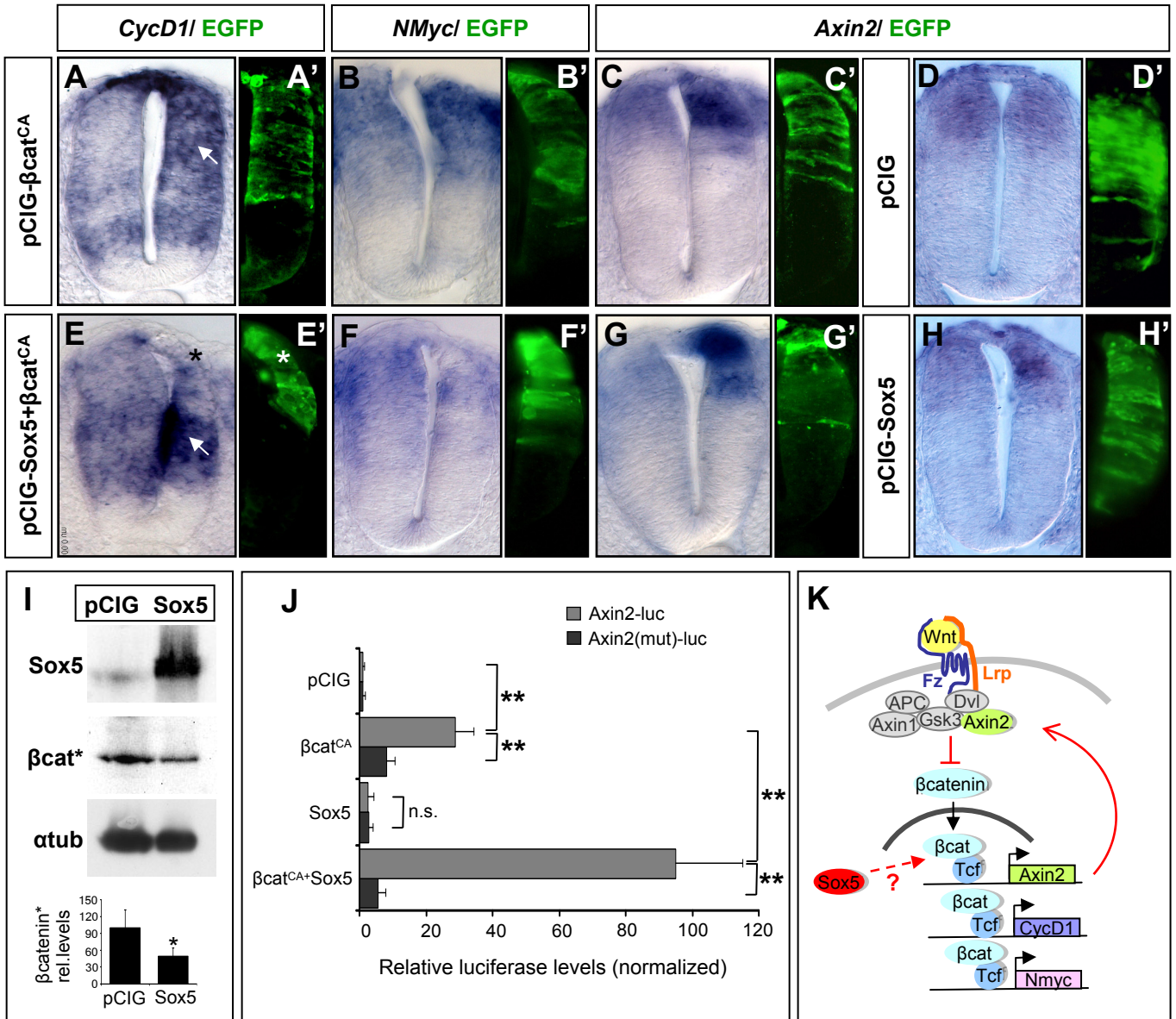
Fig 5 | Downregulation of Sox5 expression is required for the progression of dorsal interneuron differentiation. (**A-D'**) Sustained elevation of Sox5 in HH14-16 embryos induces ectopic activation of Lhx1/5 (arrows, **B,B'**) and p27^{kip1} (arrows **in D,D'**) in neurons before they reach the mantle zone, with respect to control pCIG cells (**A,A',C,C'**). (**E,F,K**) At stage HH18-22, there was a reduction in the number of Lhx1/5^{+} (**F,F'**), Brn3a^{+}, En1^{+} and Isl1/2^{+} interneurons (**K**). (**G,I,L**) At stage HH18-22,
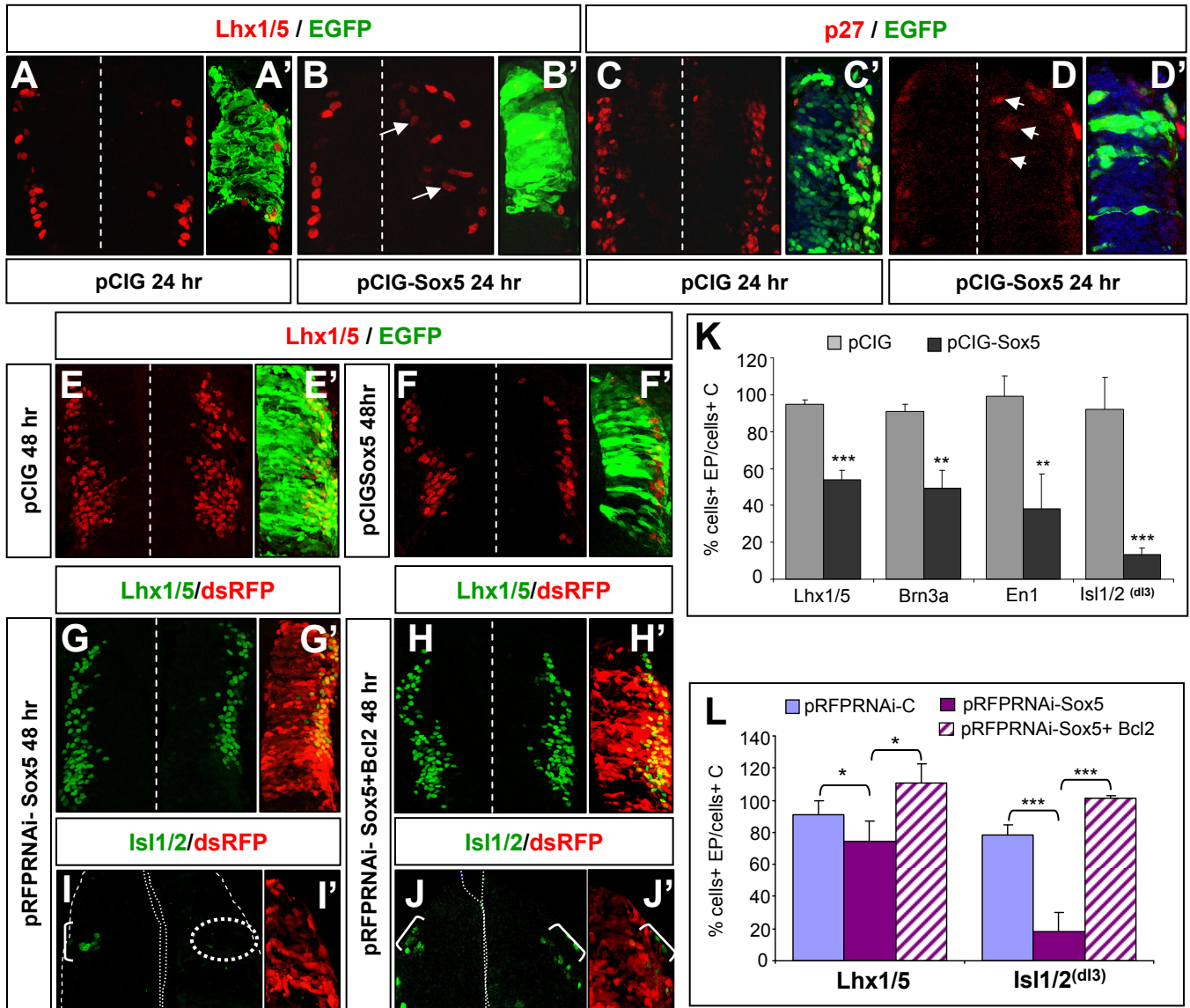
knocking down Sox5 expression (mi2, red) affects the number of Lhx1/5$^+$ (**G,G',L**) and Isl1/2$^+$ interneurons (**I,I',L**). (**H-J,L**) Coelectroporation with Bcl2 rescues the total number of Lhx1/5$^+$ (**K**) and Isl1/2$^+$ interneurons (brackets in **L**) Quantification of the number of cells expressing a given neuronal marker 48 hours PE with the indicated construct. (*) $p < 0.05$; (**) $p < 0.003$; (***) $p < 0.001$.

Sox5

A — HH10

B — HH14

Sox5/HuC/D

C — HH24 — E-I, J-L

D

Sox5/Pax7

Sox5/Isl1/2

HH24

E

F

G

H

I

J

K

L

CycD1/ EGFP

CyclinD1/RFP

pCIG-Sox5, 24 PE

pRFPRNAi-Sox5,48 PE

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).

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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
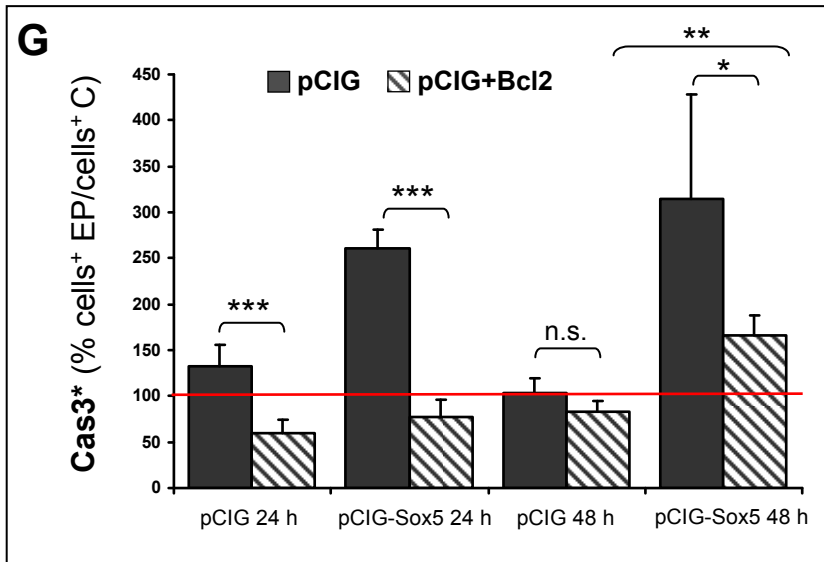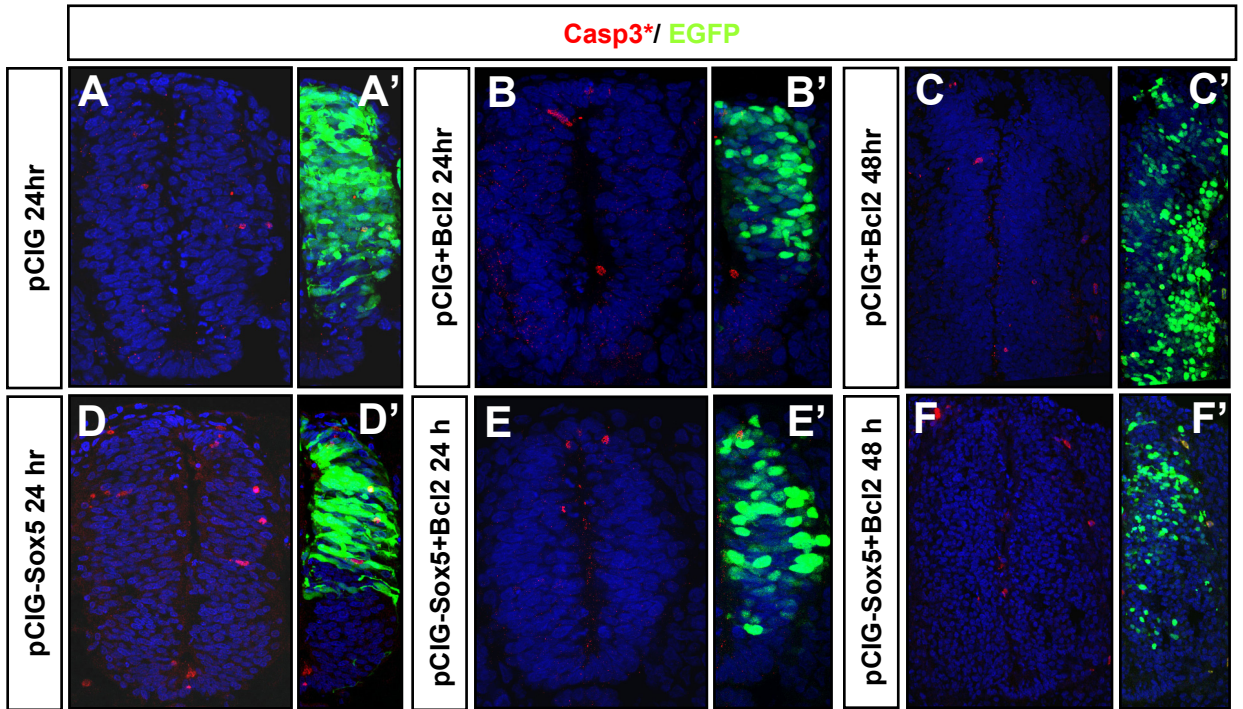
levels of apoptosis induced by pRFPRNAi-Control and pRFPRNAi-Sox5, mi2 electroporation. (**E,F**) Flow cytometry analysis of cell cycle phase distribution of cells electroporated with pRFPRNAi-Control (**E**) or pRFPRNAi-Sox5, mi2 (**F**), in combination with pCIG and the survival factor Bcl2. Knocking 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.
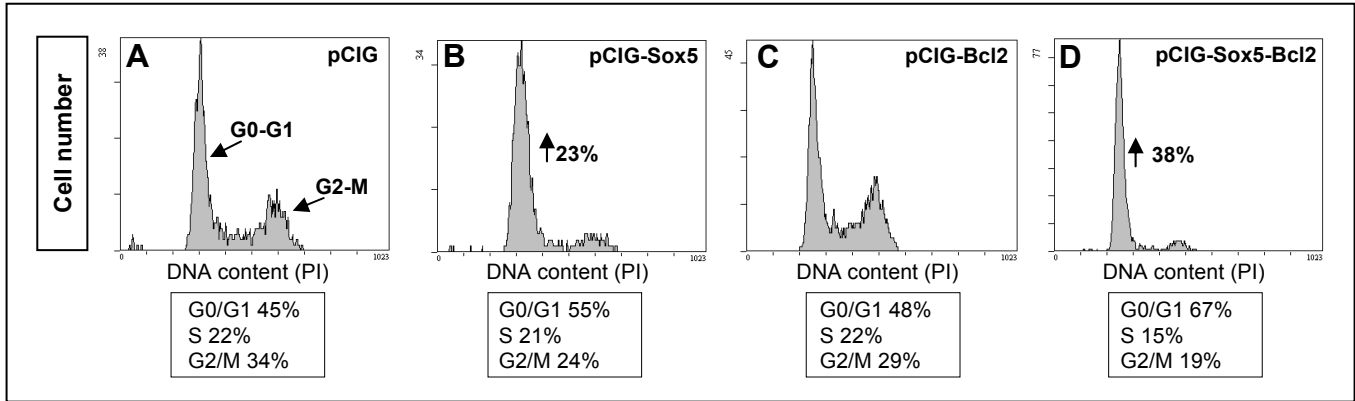
SFig 4 | Sox5 promotes cell cycle arrest by interfering with β-catenin proliferating activity. (**A-D**) Flow cytometry analysis of cell cycle progression in cells transfected with the indicated constructs. pCIG-β-catenin^{CA} promotes a 41% increase in the proportion of neuroepithelial cells in G2/M (**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**).
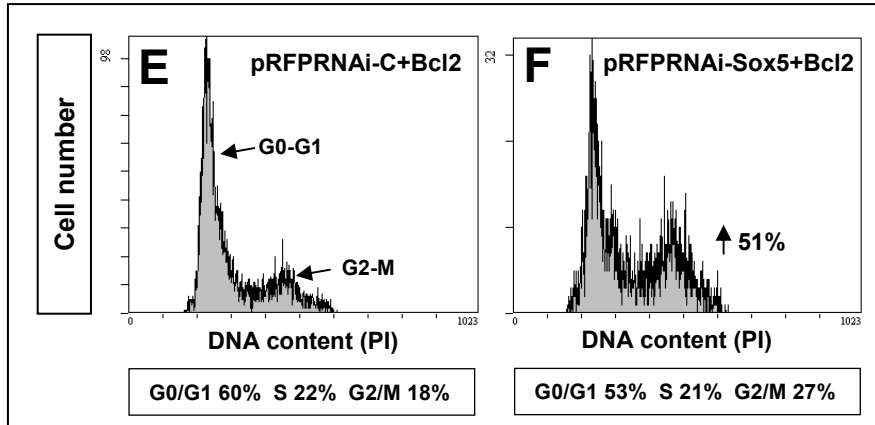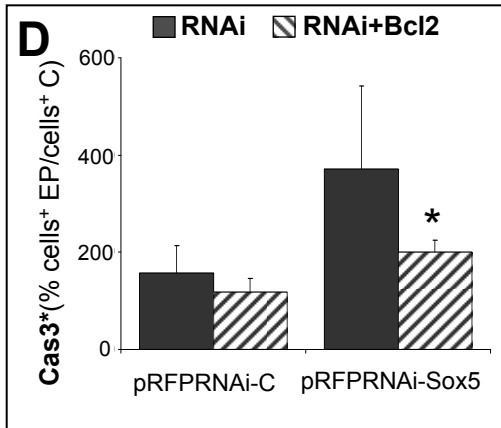
SFig 5 | Sox5 interferes with β-catenin transcriptional activity in the spinal cord.(**A**) Quantitative analysis of the transcriptional activity of Sox5 alone or in combination with β-catenin^{CA} on a Tcf (TOPFlash) transcriptional reporter in HH11-3 electroporated neural tubes. Mutations in the six 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 — A / A'
pCIG+Bcl2 24hr — B / B'
pCIG+Bcl2 48hr — C / C'
pCIG-Sox5 24 hr — D / D'
pCIG-Sox5+Bcl2 24 h — E / E'
pCIG-Sox5+Bcl2 48 h — F / F'

G

Cas3* (% cells$^+$ EP/cells$^+$ C)

■ pCIG   ▨ pCIG+Bcl2

450
400
350
300
250
200
150
100
50
0

pCIG 24 h — ***
pCIG-Sox5 24 h — ***
pCIG 48 h — n.s.
pCIG-Sox5 48 h — * , **

Cell number

A pCIG

G0-G1

G2-M

DNA content (PI)

G0/G1 45%
S 22%
G2/M 34%

B pCIG-Sox5

↑23%

DNA content (PI)

G0/G1 55%
S 21%
G2/M 24%

C pCIG-Bcl2

DNA content (PI)

G0/G1 48%
S 22%
G2/M 29%

D pCIG-Sox5-Bcl2

↑ 38%

DNA content (PI)

G0/G1 67%
S 15%
G2/M 19%

A pRFPRNAi-control pRFPRNAi-Sox5

Relative values

Sox5 expression

** 34%

Sox5 / dsRFP

B B' C C'

pRFPRNAi- Sox5, mi1   pRFPRNAi- Sox5, mi2

D ■RNAi ▨RNAi+Bcl2

Cas3*(% cells⁺ EP/cells⁺ C)

pRFPRNAi-C   pRFPRNAi-Sox5

*

E pRFPRNAi-C+Bcl2

Cell number

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%

G0/G1 44%
S 21%
G2/M 35%

G0/G1 35%
S 16%
G2/M 49%

G0/G1 50%
S 17%
G2/M 33%

G0/G1 24%
S 20%
G2/M 56%

A TOPFlash Luc Reporter (LRU)

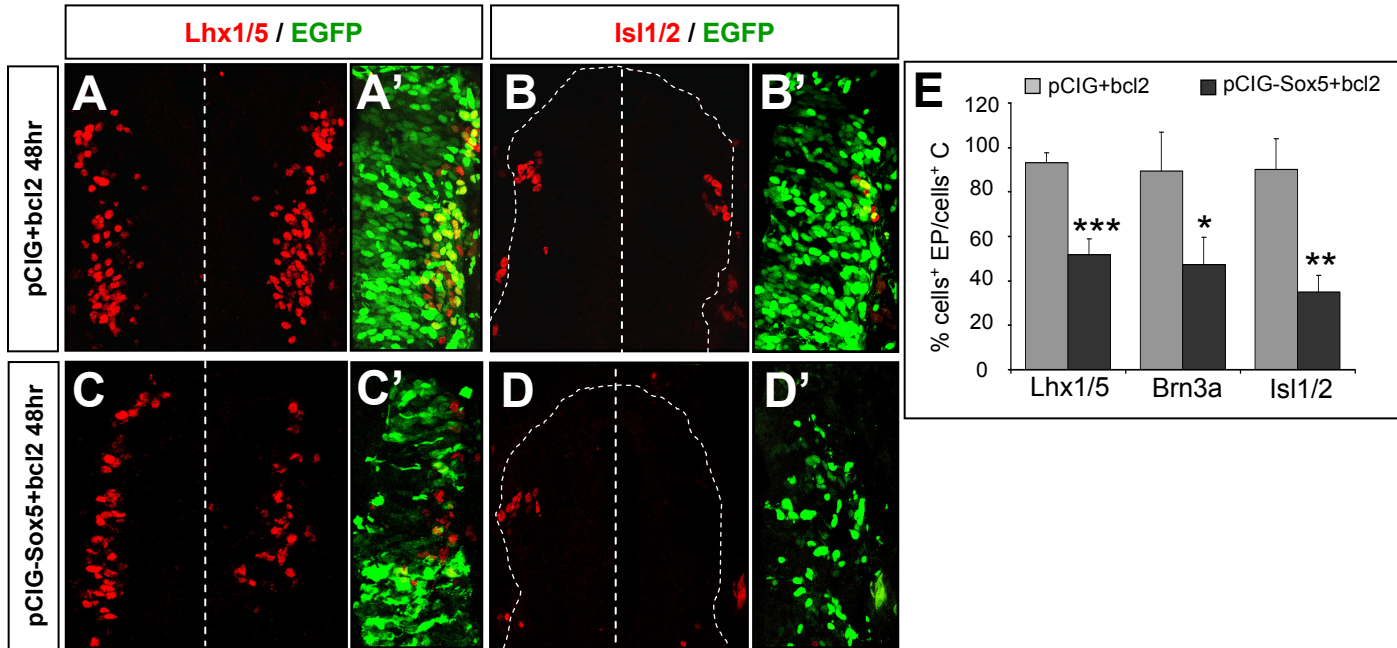**Table S1**. Analysis of hemitube thickness and cell density(cells/ µm²) in HH14-17 chick embryos electroporated with pCIG or pCIG-Sox5.

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