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Hedgehog activation is required upstream of Wnt signalling to control neural progenitor proliferation

Roberto Alvarez-Medina[1], Gwenvael Le Dreau[1], Marian Ros[2] and Elisa Martí[1,*]

The canonical Wnt and sonic hedgehog (Shh) pathways have been independently linked to cell proliferation in a variety of tissues and systems. However, interaction of these signals in the control of cell cycle progression has not been studied. Here, we demonstrate that in the developing vertebrate nervous system these pathways genetically interact to control progression of the G1 phase of the cell cycle. By in vivo loss-of-function experiments, we demonstrate the absolute requirement of an upstream Shh activity for the regulation of Tcf3/4 expression. In the absence of Tcf3/4, the canonical Wnt pathway cannot activate target gene expression, including that of cyclin D1, and the cell cycle is necessarily arrested at G1. In addition to the control of G1 progression, Shh activity controls the G2 phase through the regulation of cyclin E, cyclin A and cyclin B expression, and this is achieved independently of Wnt. Thus, in neural progenitors, cell cycle progression is co-ordinately regulated by Wnt and Shh activities.

KEY WORDS: Beta-catenin, Cell cycle, Gli proteins, Neural development, TCF transcription factors, Mouse, Chick

INTRODUCTION

The vertebrate early embryonic neural tube is an excellent model with which to investigate the proliferation/differentiation balance, as it is composed of proliferating neural precursors and terminally differentiating neurons. However, the mechanisms controlling proliferation remain poorly understood. The canonical Wnt pathway regulates the growth of neural tissue (Chen and Walsh, 2002; Megason and McMahon, 2002; Machon et al., 2003; Zechner et al., 2003; Panhuysen et al., 2004), most likely through transcriptional control of key regulators of the G1/S transition of the cell cycle, such as cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999). In addition, increasing experimental data support a proposal that the sonic hedgehog (Shh) pathway plays a major role in neural progenitor proliferation (Chiang et al., 1996; Litingtung and Chiang, 2000; Wijgerde et al., 2002; Jeong and McMahon, 2005; Cayuso et al., 2006; Locker et al., 2006), and that cyclin D1 expression is also regulated by the activity of Shh, both in the developing cerebellum (Oliver et al., 2003; Kenney and Rowitch, 2000; Kenney et al., 2003) and in the neural tube (Ishibashi and McMahon, 2002; Lobjois et al., 2004; Cayuso et al., 2006).

The opposite expression of Wnt and Shh ligands, with Wnts being largely expressed dorsally and Shh being largely restricted to the floor plate cells of the ventral-most CNS, suggested a simplistic model in which these two pathways act in parallel on different precursor populations, i.e. Wnts dorsally and Shh ventrally. Alternative models propose that these two pathways act within the same precursor cell population, either controlling different cell cycle regulators or interacting upstream of the transcriptional control of key cell cycle regulators.

Taking advantage of temporally controlled chick in ovo electroporation, together with genetic tools for the activation or repression of either pathway, we have searched for an interaction between canonical Wnt activity and the Shh pathway in the regulation of cell cycle progression of neural cells. We demonstrate that the Wnt-mediated expression of cyclin D1 requires an upstream Shh activity that includes control of *Tcf3/4* gene expression. Additionally, we show that the Shh/Gli pathway regulates G2 length and the expression of late cyclins. Analogous regulation might occur during normal growth of many other tissues and perhaps also in tumours in which a contribution by both pathways has been reported.

[1]Instituto de Biología Molecular de Barcelona, CSIC, Parc Cientific de Barcelona, C/Baldiri i Reixac 21, Barcelona 08028, Spain. [2]Instituto de Biomedicina y Biotecnología de Cantabria (CSIC-UC-IDICAN), Santander 39011, Spain.

*Author for correspondence (emgbmc@ibmb.csic.es)

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MATERIALS AND METHODS

DNA constructs

DNAs encoding the following were inserted into pCIG (Megason and McMahon, 2002): mouse Wnt1 and Wnt3a; an active form of β-catenin, β-catenin^{CA} (Tetsu and McCormick, 1999); dominant-negative forms of TCF proteins that lack the β-catenin-binding domain, Tcf1^{DN}, Tcf3^{DN} and Tcf4^{DN} (Kim et al., 2000; Tetsu and McCormick, 1999); the HMG box DNA-binding domain of TCF fused to the repressor domain of Engrailed protein, Tcf3^{EnR}, or to the VP16 transactivator, Tcf^{VP16} (Kim et al., 2000); a mutant form of patched 1 (mPtc1$^{\Delta loop2}$) (Briscoe et al., 2001); a deleted form of human GLI3 (Gli3^{R}) (Persson et al., 2002); the complementary Gli3^{Act} (Stamataki et al., 2005); a mutant version of Lrp6 acting as a dominant-negative co-receptor for the Wnt pathway (Tamai et al., 2000); and a full-length cyclin D1 (Lobjois et al., 2008). A human *BCL2* coding sequence inserted into the pcDNA3 expression vector (Yuste et al., 2002) was used for co-electroporation with Gli3^{R} to avoid reported apoptosis (Cayuso et al., 2006).

Mouse and chick embryos and in ovo electroporation

Mice heterozygous and homozygous for the *Shh*-null allele were as published (Chiang et al., 1996). Eggs from White Leghorn chickens were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951).

Chick embryos were electroporated with Clontech-purified plasmid DNA at 0.5-2 μg/μl in H_2O containing 50 ng/ml Fast Green. Briefly, plasmid DNA was injected into the lumen of HH10-12 neural tubes, electrodes were placed either side of the neural tube and electroporation carried out using an Intracel Dual Pulse (TSS10) electroporator delivering five 50-millisecond square pulses of 30-40 V.

Transfected embryos were allowed to develop to the required stages, then dissected, fixed and processed for immunohistochemistry, in situ hybridisation, luciferase assay or FACS analysis. For bromodeoxyuridine (BrdU) labelling, 5 μg/μl BrdU was injected into the neural tubes 30 minutes prior fixation.

Immunohistochemistry and in situ hybridisation

Embryos were fixed 2-4 hours at 4°C in 4% paraformaldehyde (PFA), rinsed and sectioned. Immunostaining followed standard procedures. For BrdU detection, sections were incubated in 2M HCl for 30 minutes followed by 0.1 M $Na_2B_4O_7$ (pH 8.5) rinses and anti-BrdU incubation. Antibodies were anti-GFP (Molecular Probes) and anti-BrdU (G3G4 from DSHB). Cell counting was performed on 10-40 different sections of at least five different embryos for each experimental condition.

For in situ hybridisation, embryos were fixed overnight at 4°C in 4% PFA, rinsed and processed for whole-mount RNA in situ hybridisation following standard procedures using probes for chick *Tcf3*, *Tcf4*, cyclin D1, cyclin D2, cyclin E1, cyclin E2, cyclin B2, cyclin B3 and cyclin A2 (from the chicken EST project, UK-HGMP RC). Mouse *Tcf3* and *Tcf4* probes were as published (Lei et al., 2006).

Fluorescence-activated cell sorting (FACS)

Chick embryos were electroporated at HH10/12 with the indicated DNAs and the neural tube dissected out 24 hours later. A single-cell suspension was obtained by incubation for 10-15 minutes in trypsin-EDTA (Sigma). At least three independent experiments were analysed by FACS for each experimental condition.

Hoescht and GFP fluorescence were determined by flow cytometry using a MoFlo flow cytometer (DakoCytomation, Fort Collins, CO, USA). DNA analysis (Ploidy analysis) on single fluorescence histograms was performed using Multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

In vivo luciferase reporter assay

Transcriptional activity assays of distinct components of the Shh/Gli and β-catenin/TCF pathways were performed in vivo. Chick embryos were electroporated as described above with the indicated DNAs together with a cyclin D1 luciferase reporter construct containing five TCF binding sites (Tetsu and McCormick, 1999), and a *Renilla* luciferase reporter construct carrying the CMV immediate early enhancer promoter (Promega) for normalisation. Embryos were harvested after 24 hours incubation in ovo and GFP-positive neural tubes were dissected and homogenised in Passive Lysis Buffer (Alvarez-Rodríguez et al., 2007). Firefly and *Renilla* luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega).

Statistical analysis

Quantitative data were expressed as mean±s.d. Significant differences among groups were examined by Fisher´s test.

RESULTS

Wnt activity regulates cell cycle progression throughout the dorsoventral axis of the neural tube

Even though expression of Shh ligand is restricted to the ventral-most floor plate cells (Martí et al., 1995), blocking the Shh pathway at either the receptor or the transcriptional level results in cell cycle arrest throughout the dorsoventral (DV) axis of the neural tube (NT) (Cayuso et al., 2006).

Dorsally expressed members of the Wnt family regulate proliferation of spinal cord progenitors by acting through the canonical β-catenin/TCF pathway (Megason and McMahon, 2002). To test the DV requirement of Wnt activity, we blocked the canonical Wnt pathway either at the membrane level with a dominant-negative form of the co-receptor Lrp6 ($Lrp6^{\Delta C}$) (Tamai et al., 2000), or at the transcriptional level with dominant-negative forms of Tcf1, Tcf3 and Tcf4 (Tcf^{DN}) (Alvarez-Medina et al., 2008; Kim et al., 2000) (Fig. 1A). To allow informative comparisons between embryos and constructs, we standardised the conditions used in all experiments: HH11/12 chick embryos were electroporated with each construct and following incubation and processing, analysis was restricted to the forelimb and the anterior thoracic regions. At 24 hours post-electroporation (hpe), all constructs caused a net tissue reduction owing to a uniform inhibition of proliferation along the DV axis. This was quantified as the reduction in GFP^+ cells that were $BrdU^+$ at the dorsal or ventral NT, between which there was no significant difference (Fig. 1B). Furthermore, the cell-autonomous reduction of $BrdU^+$ cells was proportional to the reported repressor capacity of each construct (Alvarez-Medina et al., 2008) (Fig. 1B-F; see Table S1 in the supplementary material). In vivo reporter analyses have shown that the Wnt pathway is active throughout the DV axis of the early developing chick and mouse NT (Megason and McMahon, 2002; Yu et al., 2008). Our results showed that loss of Wnt receptor ($Lrp6^{\Delta C}$) resulted in the cell-autonomous inhibition of proliferation even in ventral cells, indicating the long-range availability of Wnt ligands and their requirement to regulate the proliferation of neural precursors.

In a population of asynchronously cycling cells, the fraction of cells in a given phase of the cell cycle is proportional to the length of that phase and is relative to the total length of the cell cycle. To gain further insight into the cell cycle kinetics, embryos electroporated at HH11/12 were dissected 24 hpe and single-cell suspensions processed for flow cytometry analysis. The cell cycle phase distribution showed a significantly elevated percentage of transfected cells at the G1/G0 phase, with fewer cells at the S phase (Fig. 1G,H; see Table S1 in the supplementary material). Since embryo manipulation was performed at a developmental stage at which the proportion of terminally differentiated cells is still low, this result should reflect changes in G1 rather than G0. Thus, the repression of Wnt target genes lengthens the G1 phase of the cell cycle, similar to what we observed previously after inhibition of the Shh pathway (Cayuso et al., 2006), suggesting that these two pathways might interact to control progression of the G1 phase of the cell cycle.

The fact that both Wnt and Shh activities are required throughout the DV axis argues against a simplistic model in which dorsally expressed Wnts control proliferation of dorsal progenitors and ventrally expressed Shh regulates proliferation of ventral progenitors. Therefore, we sought to test for a possible genetic interaction between these two pathways in the control of cell cycle progression.

Shh signalling is required for Wnt regulation of cell cycle progression upstream of TCF

Shh signals by binding to the multi-pass transmembrane receptor patched (Ptc). In the absence of Shh, Ptc suppresses the activity of a second transmembrane protein, smoothened (Smo) (Lum and Beachy, 2004; Jiang and Hui, 2008). To inhibit Shh signalling at the receptor level, we used a mutant form of mouse (m) Ptc1 ($mPtc1^{\Delta loop2}$) that has lost the capacity to bind Shh but retains the ability to inhibit Smo and to inhibit downstream events (Briscoe et al., 2001). Electroporation of $mPtc1^{\Delta loop2}$ resulted in a cell-autonomous reduction of $BrdU^+$ cells (Fig. 2A,E; see Table S1 in the supplementary material) (Cayuso et al., 2006). To investigate a possible genetic interaction between the Wnt and Shh pathways in the regulation of proliferation, we activated the canonical Wnt pathway at different levels in a loss-of-function background for Shh activity. Transfection of the proliferative ligands Wnt1/Wnt3a, or of a stabilised form of β-catenin ($\beta\text{-catenin}^{CA}$), or a chimeric transcriptional activator form containing the HMG box DNA-binding domain of Tcf3 fused to the VP16 transactivator domain ($Tcf3^{VP16}$) (Megason and McMahon, 2002; Tetsu and McCormick, 1999; Alvarez-Medina et al., 2008), significantly increased the rate of BrdU incorporation and caused a net overgrowth of the neural tissue (Fig. 2D,E; see Table S1 in the supplementary material). However, co-electroporation of $mPtc1^{\Delta loop2}$ together with Wnt1/Wnt3a or $\beta\text{-catenin}^{CA}$ resulted in reduced BrdU incorporation

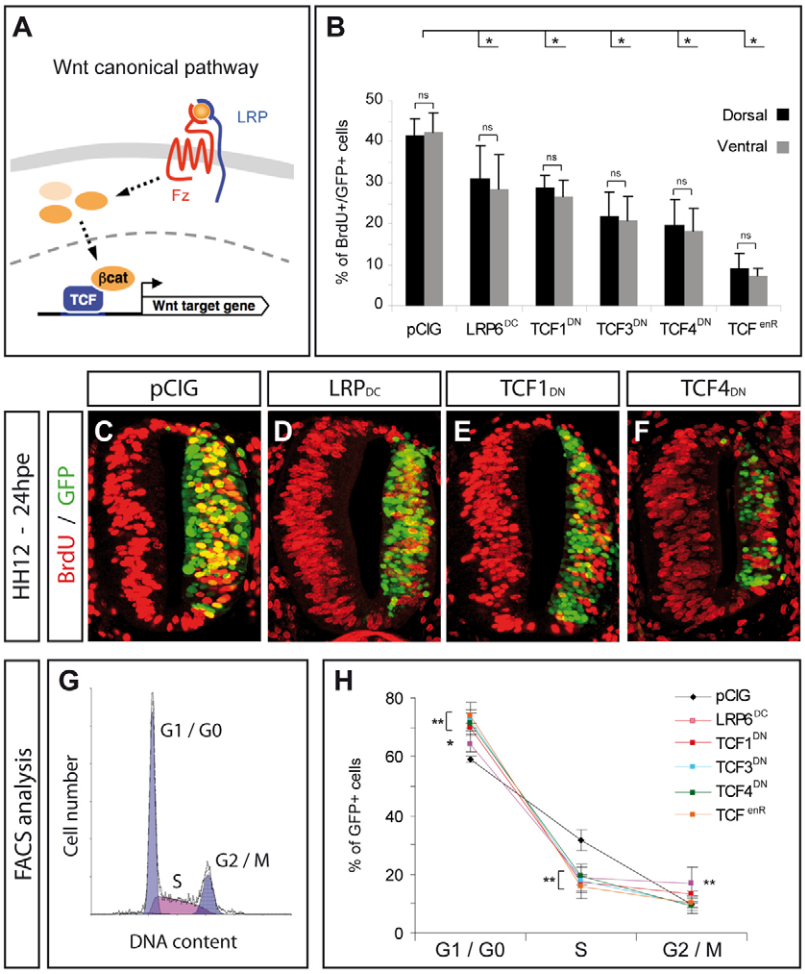

Fig. 1. Canonical Wnt activity is required for proliferation throughout the DV axis of the neural tube. (**A**) Schematic representation of components of the canonical Wnt pathway. Fz, frizzled. (**B**) Quantitative analysis of GFP-expressing cells (%) that have incorporated BrdU after a 30-minute BrdU pulse. All constructs significantly reduced the number of BrdU^{+} cells compared with the control (pCIG). Cell counting in dorsal and ventral halves of the neural tube (NT) showed no significant (ns) differences. *P≤0.0001. (**C-F**) HH11/12 chick embryos electroporated with the indicated DNAs, analysed by immunostaining for BrdU (red) and GFP (green). (**G**) Flow cytometry analysis of cell cycle phase distribution. Shown is a representative example of the cell cycle profile of cells expressing control DNA. (**H**) Quantitative analysis of cell cycle phase distribution after electroporation of the indicated DNAs. Loss of Wnt activity resulted in a significantly increased percentage of cells at the G1/G0 phase, at the expense of cells at the S phase. Note that Lrp6 caused a smaller, although significant, increase in cells at G1/G0, compensated by a significant increase in G2 cells. *P≤0.01, **P≤0.0001.

and a phenotype undistinguishable from that caused by mPtc1$^{\Delta loop2}$ alone (Fig. 2B,E; see Table S1 in the supplementary material). Interestingly, under the same loss-of-function condition for Shh, electroporation of the β-catenin-independent activator Tcf3^{VP16} rescued the BrdU-incorporation rate and recovered the normal size of the NT (Fig. 2E; see Table S1 in the supplementary material). Quantitative analysis of BrdU incorporation showed that Tcf3^{VP16} can overcome the mPtc$^{\Delta loop2}$-mediated inhibition of BrdU incorporation (Fig. 2E), although it could not enhance BrdU incorporation, as it does when endogenous levels of Shh activity are present. This suggested that Shh signalling is epistatic to the Wnt regulation of cell cycle progression (Fig. 2E).

FACS analysis of the distribution of transfected cells at the different phases of the cell cycle showed that electroporation of mPtc1$^{\Delta loop2}$ alone significantly increased the percentage of cells in G1/G0 (Fig. 2F), as previously reported (Cayuso et al., 2006). As suggested by the BrdU-incorporation analysis, co-electroporation of mPtc1$^{\Delta loop2}$ with either Wnt1/Wnt3a or β-catenin^{CA} resulted in a similar cell cycle phase distribution to that obtained after electroporation of mPtc1$^{\Delta loop2}$ alone (see Table S1 in the supplementary material). However, co-electroporation of mPtc1$^{\Delta loop2}$ with Tcf3^{VP16} resulted in a similar percentage of cells in G1/G0 as in the control, although there was a significant increase in cells in the late phases of the cell cycle (S and G2/M) (Fig. 2F; see Table S1 in the supplementary material). By contrast, activation of the Wnt pathway by electroporation of either Wnt1/Wnt3a, β-catenin^{CA} or Tcf3^{VP16} resulted in significantly fewer cells at G1/G0 as compared with the control (Fig. 2F; see Table S1 in the supplementary material). These data indicated that TCF-mediated transcriptional activation can overcome the G1 arrest caused by the loss of Shh activity, although it cannot restore the normal cell cycle profile. Instead, a significantly higher proportion of cells appear in the late phases of the cycle (Fig. 2F).

Shh activity is required for Wnt-mediated cyclin D1 expression

D-type cyclins drive progression through the G1 phase of the cell cycle, and transcriptional regulation of D-type cyclins in neural tissue depends on growth factors including Wnts and Shh (Megason and McMahon, 2002; Cayuso et al., 2006). We next analysed expression of cyclin D1 (Ccnd1) after activation of either the Wnt or Shh pathway by electroporation of dominant-active forms of β-catenin, Tcf3 or Gli3 (β-catenin^{CA}, Tcf3^{VP16} or Gli3^{Act}). In the developing NT, activation of either pathway resulted in the ectopic expression of Ccnd1 throughout the DV axis (Fig. 3A; see Fig. 6A) (Cayuso et al., 2006; Megason and McMahon, 2002). Interestingly, loss of Shh activity reduced expression of Ccnd1 (Fig. 3B) (Cayuso et al., 2006), and, in a loss-of-function background for Shh activity, β-catenin^{CA} lost, whereas Tcf3^{VP16} retained, the capacity to activate Ccnd1 expression (Fig. 3C,D). These in situ data link G1 arrest with the regulation of Ccnd1 expression.

To gain further insight into the requirement and conservation of the Shh and Wnt pathways in the activation of Ccnd1 expression in neural cells, we utilised the human 1.7 kb *CCND1* promoter that

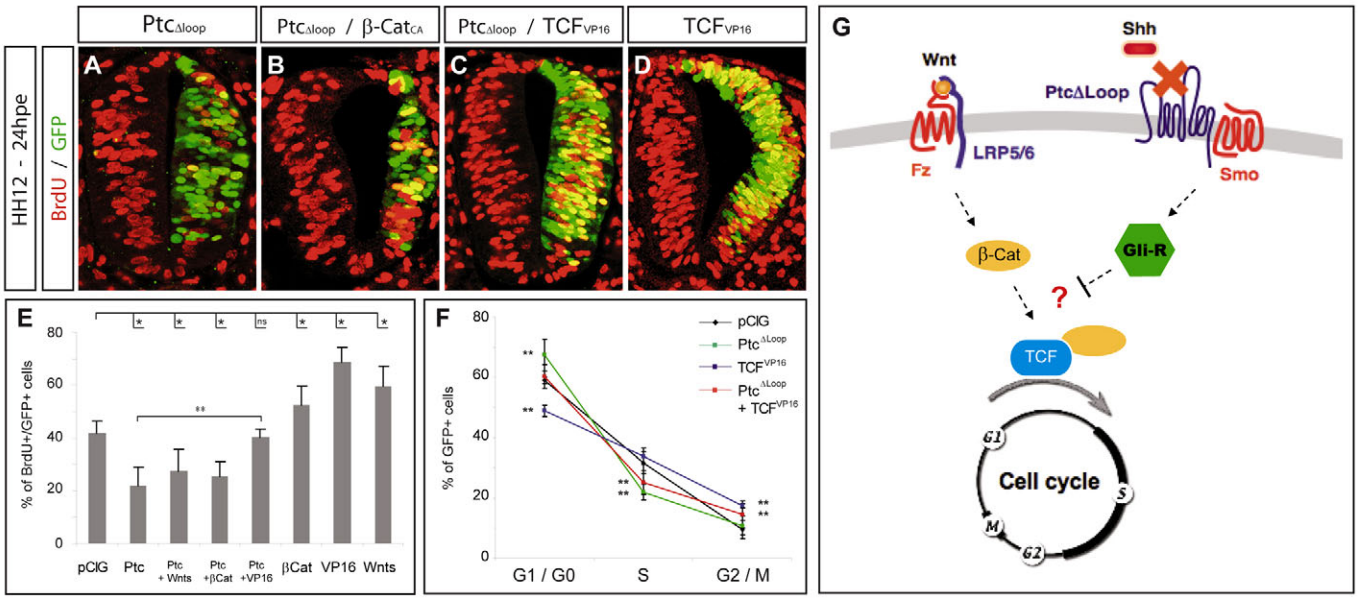

Fig. 2. Shh activity is required for Wnt-mediated cell cycle progression. (**A-D**) HH11/12 chick embryos were electroporated with the indicated DNAs and analysed at 24 hpe by immunostaining for BrdU (red) and GFP (green). (**E**) Quantitative analysis of GFP-expressing cells that have incorporated BrdU after a 30-minute BrdU pulse. All treatments result in statistically significant differences to the control (pCIG), except for the combination mPtc$^{\Delta Loop2}$ + Tcf3^{VP16} (ns). *P≤0.0001. (**F**) Quantitative analysis of cell cycle phase distribution after electroporation of the indicated DNAs. Loss of Shh activity resulted in a significant increase in the percentage of cells arrested at the G1/G0 phase, at the expense of S/G2 cells. Tcf^{VP16} co-electroporation resulted in a significant decrease in the percentage of cells in G1/G0. Co-electroporation experiments show rescue of the percentage of cells in G1, but not of S/G2 cells. **P≤0.0001. (**G**) Schematic representation of Shh and Wnt activities in the regulation of the G1 cell cycle progression of neuroepithelial cells.

contains five consensus TCF binding sites for transient luciferase reporter assays (Tetsu and McCormick, 1999). Endogenous levels of this reporter appeared to be very low in chick NT cells, and therefore insufficient to reproduce the inhibition of Ccnd1 expression observed after electroporation of mPtc$^{\Delta Loop2}$ (Fig. 3E). However, in NT cells, β-catenin^{CA} was a potent activator of the reporter as previously published (Tetsu and McCormick, 1999), and this activation was significantly reduced by co-electroporation with mPtc1$^{\Delta loop2}$ (Fig. 3E). Tcf3^{VP16} also had the capacity to activate this reporter in NT cells, although at lower levels than with β-catenin^{CA} (Fig. 3E). Consistent with the in situ data, the Tcf3^{VP16}-mediated activation of the reporter was not significantly inhibited by co-electroporation with mPtc1$^{\Delta loop2}$ (Fig. 3E).

Even though *Ccnd1* is a direct target gene of the Wnt canonical pathway, and active TCF consensus binding sites are present in the *Ccnd1* promoter, these data indicate that Shh activity is required in vivo for proper Ccnd1 expression, upstream of TCF transcriptional activation (Fig. 3F).

Shh activity is required for the expression of Tcf3/4

Our results suggested that Shh signalling might transcriptionally regulate some gene or genes required for Wnt-mediated expression of Ccnd1 and for cell cycle control, the simplest explanation being a putative regulation of TCF gene expression by the Shh pathway.

To test this hypothesis, chick embryos electroporated with mPtc1$^{\Delta loop2}$ were analysed by in situ hybridisation for the expression of the two TFC genes that are more widely expressed throughout the DV axis of the NT, i.e. *Tcf3* and *Tcf4* (Alvarez-Medina et al., 2008). Blockade of Shh signalling caused a reduction in *Tcf3* and *Tcf4* expression levels, concomitant with the reported size reduction of the transfected side (Fig. 4A,B). Conversely, ectopic activation of the Shh pathway by electroporation of Gli3^{Act} resulted in the ectopic expression of *Tcf3* and *Tcf4* (Fig. 4C,D) and the previously reported net overgrowth of the NT (Cayuso et al., 2006). Moreover, we took advantage of *Shh* knockout mice that generate a genetically consistent Shh loss-of-function background (Chiang et al., 1996). In E9.5 mouse embryos, NTs showed a clear reduction in *Tcf3* and *Tcf4* expression as compared with wild-type littermates (Fig. 4E-H).

To test whether Shh signalling was directly regulating *Tcf3* and *Tcf4* expression at the transcriptional level, we searched for highly conserved non-coding DNA regions (HCNRs) within the human *TCF3* and *TCF4* loci that could function as potential enhancer modules. Although poorly annotated, both genes showed upstream, intronic and downstream HCNRs among widely divergent vertebrate species including human, mouse, chick, *Xenopus* and *Fugu*; *Tcf4*, in particular, contains a large number of such regions (VISTA or ECR browser). However, we could not find the core consensus Gli binding sequence [5′-GACCAC(C/A)CA-3′ (Hallikas et al., 2006)] within any of these HCNRs. This result favoured a model in which Shh signalling regulates *Tcf3* and *Tcf4* expression indirectly. In support of this indirect regulation, two recent reports that searched for direct Gli target regions along the whole genome, although unmasking known direct Gli target genes such as *FoxA2* and *Ptc1*, did not uncover any TCF genes (Vokes et al., 2007; Vokes et al., 2008).

Shh activity regulates the expression of late cell cycle components and G2 phase length

Our results show that expression of Ccnd1 requires the integration of Shh and Wnt activities, and that loss of either growth factor results in impaired cell cycle progression.

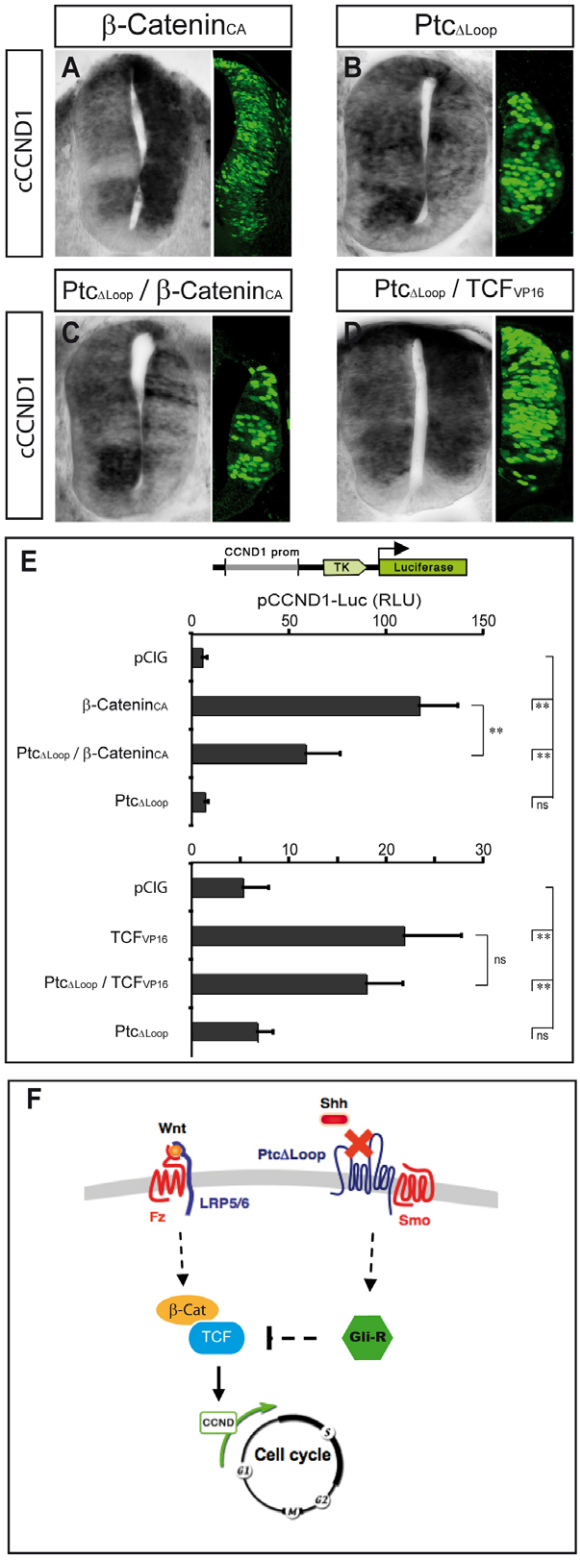

Fig. 3. The canonical Wnt pathway regulates *Ccnd1* transcription by an Shh-dependent mechanism. (**A-D**) Chick embryos were electroporated with the indicated DNAs and analysed at 24 hpe by in situ hybridisation with a chick *Ccnd1* probe. The electroporated side is on the right. GFP (green) reveals transfected cells. (**E**) In vivo quantitative analysis of the transcriptional activities of different components of the Wnt and Shh pathways on the human *CCND1* promoter. Embryos were electroporated with the indicated DNAs and analysed at 24 hpe for luciferase activity. Endogenous levels are low. β-catenin^{CA} and Tcf3^{VP16} are both sufficient to significantly transactivate the reporter, although to different levels. $^{**}P \leq 0.0001$. (**F**) Summary of Shh and Wnt activities in the regulation of Ccnd1 expression.

We next sought to establish whether the regulation of D-type cyclins for G1 progression was the sole activity at which the Wnt and Shh pathways converge to control cell cycle progression. Co-electroporation experiments included an expression vector containing Ccnd1 (Lobjois et al., 2008) together with the strongest constitutive transcriptional repressor forms for each signalling pathway, i.e. Tcf3^{EnR} and Gli3^{R} for Wnt and Shh, respectively (Alvarez-Medina et al., 2008; Cayuso et al., 2006). Although electroporation of Tcf3^{EnR} or Gli3^{R} caused a dramatic cell-autonomous loss of BrdU incorporation (Fig. 5A,C; see Table S1 in the supplementary material), co-electroporation with Ccnd1 was sufficient to totally rescue the loss of BrdU^{+} cells caused by Tcf3^{EnR} to control levels (Fig. 5B,F; see Table S1 in the supplementary material). Interestingly, however, Ccnd1 was insufficient to rescue the loss of BrdU incorporation caused by the repressor form Gli3^{R} (Fig. 5C,D,F; see Table S1 in the supplementary material), even though ectopic expression of Ccnd1 alone resulted in a substantial increase in BrdU^{+} cells (Fig. 5E,F; see Table S1 in the supplementary material). These data indicate that Gli transcriptional activity might be controlling the expression and/or activity of additional factors required for cell cycle progression.

To test this hypothesis, we analysed the cell cycle phase distribution at 24 hpe following electroporation with the various constructs. Ccnd1 electroporation resulted in significantly fewer cells at the G1/G0 phase, at the expense of an increased proportion of cells at the late S and G2/M phases. Conversely, electroporation of Tcf3^{EnR} resulted in more cells at the G1/G0 phase, at the expense of fewer cells at S phase (Fig. 5G). Co-electroporation of Ccnd1 and Tcf^{EnR} resulted in a cell cycle phase distribution equivalent to that of control pCIG-electroporated cells (Fig. 5G; see Table S1 in the supplementary material), indicating that the activity of Wnt in cell cycle regulation is restricted to the control of the G1 phase. Interestingly, although electroporation of Gli3^{R} resulted in a similar cell cycle profile to that obtained after electroporation of Tcf3^{EnR}, co-electroporation with Ccnd1 did not restore the normal cell cycle profile. Co-electroporation of Ccnd1 and Gli3^{R} resulted in a similar percentage of cells in G1/G0 as in control cells, whereas there was a significantly higher proportion of cells at the G2/M phase (Fig. 5H; see Table S1 in the supplementary material).

Interestingly, blockade of the Shh pathway at either the receptor level by electroporation of mPtc1$^{\Delta loop2}$, or at the transcription level by electroporation of Gli3^{R}, resulted in the gradual reduction of Shh activity and in the concomitant gradual reduction of BrdU incorporation (Cayuso et al., 2006). In either experimental condition, progression through the G1 phase of the cell cycle was restored by co-electroporation of the dominant-active form of TCF (Fig. 2F) or by co-electroporation of Ccnd1 (Fig. 5H), although in both situations there was a significantly high proportion of cells at the late phases of the cycle. These results highlight the potential role of the Shh pathway in the regulation of late phases of the cell cycle.

To test this possibility, we performed an in situ hybridisation screen for additional cyclins expressed in the developing NT under the control of the Shh pathway. The Shh pathway was activated by

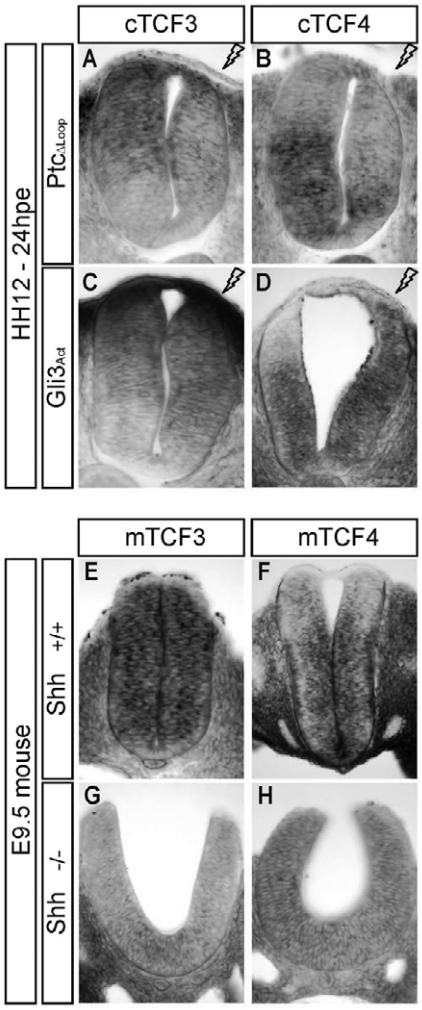

Fig. 4. Shh activity is required for the expression of *Tcf3/4*. (**A**,**B**) Chick embryos electroporated with mPtc$^{\Delta Loop2}$ show reduced expression of *Tcf3* and *Tcf4* on the electroporated side (arrowhead) as detected by in situ hybridisation. (**C**,**D**) Chick embryos electroporated with Gli3^{Act} show overgrowth of the electroporated side (arrowhead), together with ectopic expression of *Tcf3* and *Tcf4*. (**E**,**F**) Wild-type E9.5 mouse embryos show widespread expression of *Tcf3* and *Tcf4* in the NT as detected by in situ hybridisation. (**G**,**H**) *Shh*$^{-/-}$ E9.5 littermate mouse embryos show reduced expression of *Tcf3* and *Tcf4* in the open NT.

transfection of Gli3^{Act,} at HH11/12, and embryos were analysed at 24 hpe by in situ hybridisation for the expression of the various cyclins. The results showed that activation of the Shh pathway was sufficient for the overexpression of *Ccnd1/2*, *Ccne1/2*, *Ccna2/3* and *Ccnb2/3* (Fig. 6A-E; data not shown). As previously reported (Megason and McMahon, 2002), none of these late cyclins was upregulated after β-catenin^{CA} electroporation (data not shown). This analysis confirmed that the Shh pathway controls cell cycle progression of neural cells by the regulation of G1 and G2 cyclins (Fig. 6F).

DISCUSSION

In this study, we provide evidence that Wnt and Shh signalling regulate, in a cell-autonomous manner, the proliferation of progenitors throughout the DV axis of the developing NT. Moreover, our data indicate that the activity of Shh is epistatic to Wnt. By controlling the expression of the Tcf3/4 transcription factors, Shh activity is required for canonical Wnt/β-catenin transcriptional activity. This provides a genetic mechanism through which Shh and Wnt activities are integrated in the control of Ccnd1 expression and of progression through the G1 phase of the cell cycle. Additionally, Shh appeared to have multiple roles in the regulation of late cell cycle progression, controlling the expression and/or function of late cell cycle regulators.

These and previous results show that both Wnt and Shh activities are required throughout the DV axis of the developing NT, arguing against a simplistic model in which dorsally expressed Wnts control proliferation of dorsal progenitors, whereas ventrally expressed Shh regulates proliferation of ventral progenitors. Blockade of Shh signalling affects the survival and proliferation of progenitors in both dorsal and ventral regions of the NT (Cayuso et al., 2006), further supporting an extended range of influence of Shh that encompasses progenitors throughout most of the NT. This is consistent with the observation that elevated levels of the Shh-responsive gene *Ptc1* are present in a broad domain of progenitors that includes the dorsal NT (Goodrich et al., 1997), and with the fact that in embryos lacking Shh signalling, the entire NT, not just the ventral regions, appears decreased in size (Chiang et al., 1996; Litingtung and Chiang, 2000; Thibert et al., 2003; Wijgerde et al., 2002). Thus, the long-range action of Shh is required not only for the patterning of progenitors, but also for their proliferation.

Evidence that canonical Wnt/β-catenin activity is required for neuroepithelial cell proliferation comes from the introduction of loss- and gain-of-function mutations into the mouse β-catenin locus (Machon et al., 2003; Zechner et al., 2003), which show that the tissue mass of the spinal cord and several brain areas, including the cerebral cortex and hippocampus, is reduced after ablation of β-catenin. Our data now indicate that active canonical Wnt signalling is required for proliferation in both the dorsal and ventral regions of the NT. As discussed for the Shh pathway, the widespread expression of components of the canonical Wnt pathway throughout the DV axis of the NT supports a wide range of Wnt activity. Together, these data raised the question of whether these two mitogenic factors interact to ensure even growth of the NT. It is possible that the two factors regulate proliferation of the NT via distinct mechanisms involving different transcriptional responses, and our experiments cannot exclude this as an additional mechanism. However, data presented here support a genetic mechanism through which Shh and Wnt activities are integrated to control expression of the cell cycle regulator Ccnd1.

Several levels of interaction between the Shh and Wnt pathways have been proposed. On the one hand, expression of Gli3 in the dorsal NT directly depends on Wnt/TCF activity (Alvarez-Medina et al., 2008; Yu et al., 2008). This is supported by the identification of several consensus TCF binding sequences within HCNRs in the human *GLI3* locus (Abbasi et al., 2007; Alvarez-Medina et al., 2008). Two of these TCF binding sites were functionally responsive to Wnt signalling manipulation in chick NT (Alvarez-Medina et al., 2008). On the other hand, we have shown another level of genetic interaction between Shh and Wnt. By in vivo gain- and loss-of-function experiments, we show that expression of Tcf3/4 depends on Shh activity. The results suggested that in this case, although Tcf3/4 are not direct targets of Shh, their expression is nevertheless impaired in a genetic loss-of-function for Shh. Thus, both signals, expressed at the opposite DV poles of the NT, regulate complementary expression of their effectors (transcription factors) required for the transcriptional

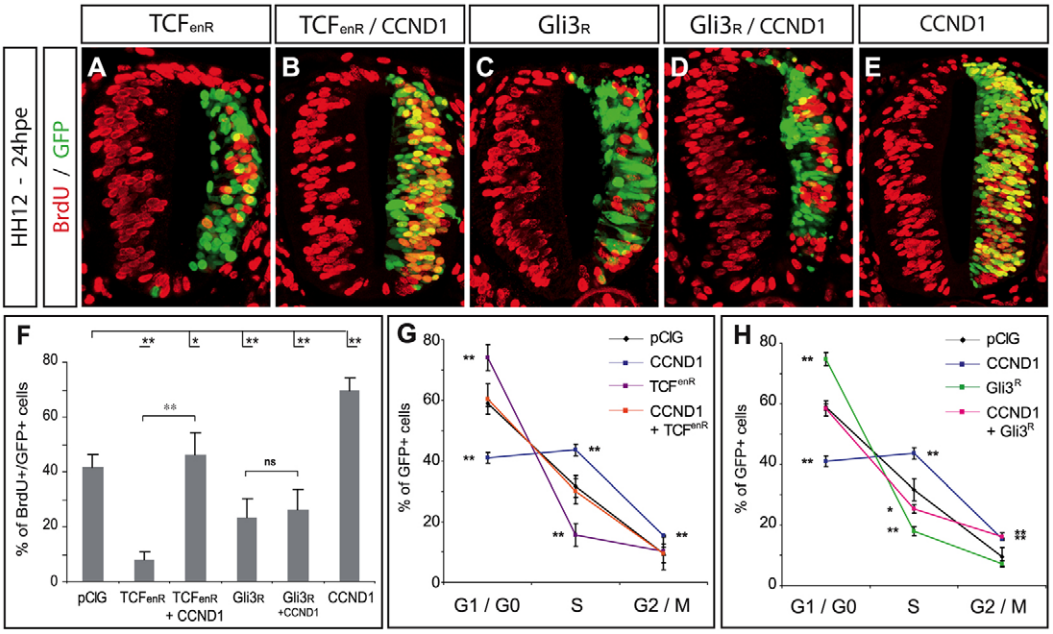

Fig. 5. Shh activity regulates the expression of late cell cycle components and G2 phase length. (**A-E**) Representative sections of chick embryos electroporated at HH11/12 with the indicated DNAs and analysed at 24 hpe for BrdU (red) and GFP expression (green). (**F**) Quantitative analysis of proliferation after a 30-minute BrdU pulse at 24 hpe with the indicated DNAs. *$P \leq 0.01$, **$P \leq 0.0001$. (**G**) Quantitative analysis of cell cycle phase distribution at 24 hpe with the indicated DNAs. Transcriptional repression of TCF target genes resulted in a significant increase in the percentage of cells at the G1/G0 phase, at the expense of cells in S phase. The percentage of cells in each phase of the cycle is rescued to a normal distribution by co-electroporation with Ccnd1. **$P \leq 0.0001$. (**H**) Transcriptional repression of Gli target genes resulted in a significantly increased percentage of cells at the G1/G0 phase, at the expense of cells in S phase. Co-electroporation with Ccnd1 restored the normal proportion of cells at the G1 phase, but led to an increased percentage of cells in G2/M, as seen for electroporation of Ccnd1 alone. *$P \leq 0.05$, **$P \leq 0.0001$.

activity of either pathway. This ensures that both the Shh and Wnt pathways can activate cell responses all along the DV axis of the developing CNS.

Additionally, the repressor form of Gli3, which is processed in the absence of Shh activity to generate a transcriptional repressor (Jacob and Briscoe, 2003), has been shown to physically interact with β-catenin, inhibiting its positive activity on TCF targets (Ulloa et al., 2007). Our in vivo results are consistent with this model because in the loss-of-function background for Shh activity generated by electroporation of $mPtc^{\Delta Loop2}$, $Gli3^R$ forms might interact with endogenous β-catenin, thus preventing interaction with TCF. However, because we show that under this genetic condition Tcf3/4 are not expressed, any influence of this proposed inhibition through $Gli3^R$-β-catenin interaction should be negligible, at least in the developing NT.

Wnt and Shh are growth factors that regulate the expression of multiple target genes in a cell-context-dependent manner. We focused our analysis on the expression of cell cycle regulators and on their consequences for the cell cycle profile of NT progenitors. *Ccnd1* is a direct target gene of the Wnt canonical pathway and active TCF consensus binding sites are present in the *Ccnd1* promoter. Surprisingly, we found that in the absence of Shh signalling, Wnt was unable to activate Ccnd1 expression. Our results show that this is the consequence of reduced TCF expression in the absence of Shh.

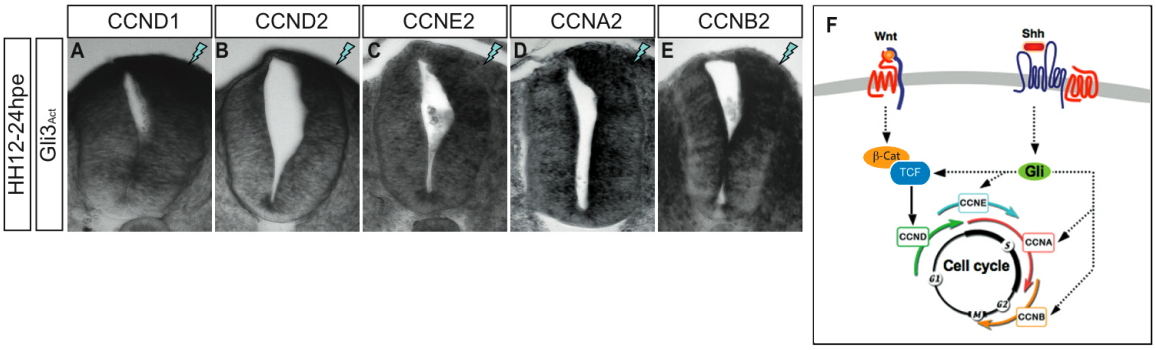

Fig. 6. In situ expression analysis of cyclins regulated by Shh signalling. (**A-E**) HH11/12 chick embryos were electroporated with the indicated DNAs and analysed at 24 hpe by in situ hybridisation with the indicated probes. Arrowhead indicates the electroporated side of the neural tube. (**F**) Summary of Shh and Wnt activities in the regulation of the G1 and G2 progression of neuroepithelial cells.

Additionally, our results show that Wnt activity is restricted to the G1 phase of the cell cycle, whereas Shh plays a role in the regulation of the late cell cycle. Expression of late cyclins depends on Shh/Gli but not on Wnt/β-catenin activities (Megason McMahon, 2002). This analysis confirmed that the Shh pathway controls cell cycle progression of neural cells through the regulation of G1 and G2 cyclins. Supporting these data, Hedgehog (Hh)-mediated regulation of the G2 phase has recently been reported in neural precursors of the developing retina (Locker et al., 2006). Moreover, these authors showed that Hh transcriptionally activates not only *Ccnd1*, but also *Ccna2*, *Ccnb1* and *Cdc25c*, which are G2 phase activators. Additionally, Shh/Gli signalling upregulates the G2/M activator Cdc25b in NT precursors (Bénazéraf et al., 2006) and the forkhead transcription factor FoxM1 in cerebellar granule neuron precursors (Schüller et al., 2007). Altogether, these data consistently support widespread regulation by Hh of the late cell cycle.

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Supplementary material
Supplementary material for this article is available at
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