

# Wnt canonical pathway restricts graded Shh/Gli patterning activity through the regulation of Gli3 expression

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Dorsoventral patterning of the vertebrate nervous system is achieved by the combined activity of morphogenetic signals secreted from dorsal and ventral signalling centres. The Shh/Gli pathway plays a major role in patterning the ventral neural tube; however, the molecular mechanisms that limit target gene responses to specific progenitor domains remain unclear. Here, we show that Wnt1/Wnt3a, by signalling through the canonical  $\beta$ -catenin/Tcf pathway, control expression of dorsal genes and suppression of the ventral programme, and that this role in DV patterning depends on Gli activity. Additionally, we show that Gli3 expression is controlled by Wnt activity. Identification and characterization of highly conserved non-coding DNA regions around the human Gli3 gene revealed the presence of transcriptionally active Tcf-binding sequences. These indicated that dorsal Gli3 expression might be directly regulated by canonical Wnt activity. In turn, Gli3, by acting as a transcriptional repressor, restricted graded Shh/Gli ventral activity to properly pattern the spinal cord.

**KEY WORDS:** Spinal cord, Neural development, Pattern formation, Wnt canonical signalling,  $\beta$ -catenin, Tcf/Lef1 transcription factors, Hedgehog signalling, Gli transcription factors, Gli3 locus, Mouse, Chick

## INTRODUCTION

Distinct types of neuron are generated along the dorsal ventral (DV) axis of the developing spinal cord. Neurons that process and relay sensory information primarily reside in the dorsal region, whereas neurons that modulate and direct motor control reside in the ventral region of the spinal cord (Ramón and Cajal, 1911). Over the past decade, developmental studies have identified the dorsal and the ventral midlines as signalling centres that instruct the generation of the appropriate types and numbers of cells along the DV axis of the neural tube. The molecular nature of these morphogenetic signals is beginning to be unravelled and includes sonic hedgehog (Shh), the Wg/Wnt proteins and the bone morphogenetic protein (Bmp) families of signalling proteins that impose (either alone or working in concert) fate and proliferative decisions to neural progenitors.

In the ventral neural tube (NT), the activity of the morphogen Shh, which is secreted from the notochord and from floor-plate cells, represents the major signalling pathway that leads to the generation of distinct classes of neurons within specific DV locations. Shh signalling, transduced into a gradient of Gli transcriptional activity, mimics all notochord and floor-plate patterning functions. Incremental changes in Shh concentration or in levels of Gli activity determine alternative neuronal subtypes by regulating the spatial pattern of expression, in ventral progenitor cells, of transcription factors that include members of the homeodomain (HD) and basic helix-loop-helix (bHLH) families (Briscoe and Ericsson, 2001; Jessell, 2000; Martí et al., 2005; Stamatakis et al., 2005; Bai et al., 2004; Bai and Joyner, 2001). The subdivision of progenitors within the ventricular zone is the initial requirement for the generation of distinct neuronal subtypes. Subsequently, the profile of HD and bHLH proteins expressed by precursor cells acts to specify the identity of neurons derived from each progenitor domain (Jessell, 2000; Martí et al., 2005).

In the dorsal NT, multiple members of the Bmp and the Wnt families are secreted from the ectoderm overlaying the neural tube and from the dorsal-most roof plate cells. Activity of these signalling proteins in DV pattern formation is not well understood, although it appears that Bmps play a major role in dorsal cell fate specification (Liu and Niswander, 2005). However, several lines of evidence have assigned a major role in the regulation of growth to Wnt proteins (Cayuso and Martí, 2005).

Wnts are a large family of highly conserved secreted signalling proteins related to the *Drosophila* wingless protein, which regulates cell-to-cell interactions during embryogenesis (<http://www.stanford.edu/~rnusse/wntwindow.html>). As currently understood, Wnt proteins bind to receptors of the frizzled family on the cell surface. Through several cytoplasmic relay components, Wnt signal is transduced through the canonical pathway to  $\beta$ -catenin, which then enters the nucleus and forms a complex with Tcfs (T-cell factors) to activate transcription of Wnt target genes (Logan and Nusse, 2004).

In the spinal cord, proteins of Wnt family have been identified as components of roof-plate signalling. Several members of the Wnt family, including Wnt1 and Wnt3a, are expressed in the dorsal midline region in both mouse and chick developing spinal cord (Hollyday et al., 1995; Megason and McMahon, 2002; Parr et al., 1993; Robertson et al., 2004). Although Wnts have primarily been considered to be mitogenic signals for neural tube cells (Dickinson et al., 1994; Megason and McMahon, 2002; Cayuso and Martí, 2005), recent studies indicate that Wnt signalling might play an additional role in cell fate specification. In particular, analysis of *Wnt1/Wnt3a* double mutant mouse embryos revealed a severe reduction in number of dorsal interneurons (dI1-3) accompanied by an increase in number of more ventrally located interneurons (Muroyama et al., 2002). Interestingly though, this seems not to be restricted to spinal cord development, as Wnt/ $\beta$  catenin activity appears also to control expression of dorsal markers and suppression of the ventral programme in the anterior CNS (Backman et al., 2005).

Here, we show members of the Tcf family of transcription factors to be differentially expressed in the developing spinal cord, with their expression domains encompassing the entire DV

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axis. Activation of the  $\beta$ -catenin/Tcf pathway by chick in ovo electroporation experiments expanded expression of dorsal markers, whereas inhibition of Wnt transcriptional activity suppressed the dorsal programme and expanded ventral gene expression. Epistatic experiments showed these phenotypes to be largely dependent on Gli activity and we show that expression of Gli3, the main repressor of the Shh/Gli pathway, is regulated by  $\beta$ -catenin/Tcf activity. In turn Gli3, by acting as a transcriptional repressor, restricts the graded Shh/Gli ventral activity to properly pattern the spinal cord. Altogether, our data indicate that Wnt signalling through an indirect mechanism was required to restrict Shh activity in the dorsal NT.

## MATERIALS AND METHODS

### DNA constructs

The following DNAs were inserted into pCIG (Megason and McMahon, 2002): full coding mouse *Wnt1*, *Wnt3a* and *Wnt4* (Megason and McMahon, 2002); full coding chick *noggin*; a mutant form of  $\beta$ -catenin lacking amino acids 29-48 and therefore lacking phosphorylation sites necessary for degradation,  $\beta$ -catenin<sup>CA</sup> (Tetsu and McCormick, 1999); dominant-negative forms of Tcf proteins that lack the  $\beta$ -catenin binding domain, Tcf1<sup>DN</sup>, Tcf3<sup>DN</sup> and Tcf4<sup>DN</sup> (Kim et al., 2000; Tetsu and McCormick, 1999); the HMG box DNA-binding domain of Tcf3 fused to the repressor domain of Engrailed protein Tcf3<sup>EnR</sup> or to the VP16 transactivator of herpes simplex virus Tcf<sup>VP16</sup> (Kim et al., 2000); the sequence encoding amino acids 325-404 of the zebrafish Tcf3 containing the HMG box DNA-binding domain Tcf<sup>HMG</sup>; a full-length human Gli3, a deleted form of Gli3 encoding amino acids 1-768, Gli3<sup>R</sup> (Persson et al., 2002); the complementary form encoding amino acids 468-1580, Gli3<sup>Act</sup> (Stamatakis et al., 2005); the sequence encoding amino acids 471-645 of the human Gli3 zinc-finger, Gli<sup>ZnF</sup> (Cayuso et al., 2006); and a dominant negative version of PKA where the cyclic AMP-binding sites of the regulatory subunit have been mutated (Epstein et al., 1996).

### Chick in ovo electroporation

Eggs from White-Leghorn chickens were incubated at 38.5°C in an atmosphere of 70% humidity and staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951).

Chick embryos were electroporated with Clontech purified plasmid DNA at 2-3  $\mu$ g/ $\mu$ l in H<sub>2</sub>O with 50 ng/ml Fast Green as reported (Cayuso et al., 2006). Transfected embryos were allowed to develop to the specific stages, then dissected, fixed and processed for immunohistochemistry or in situ hybridization.

### Wnt1<sup>-/-</sup>; Wnt3a<sup>-/-</sup> double homozygous mutant embryos

Compound heterozygotes of Wnt1<sup>+/-</sup> and Wnt3a<sup>+/-</sup> were produced by crosses between heterozygous mice carrying a null allele of Wnt1 or Wnt3a, and maintained by backcrossing to C57/Bl6 (Muroyama et al., 2002). Doubly homozygous mutants were identified among embryos derived from matings between compound heterozygotes.

### Immunohistochemistry

Embryos were fixed for 2-4 hours at 4°C in 4% paraformaldehyde in PB, and sectioned in a Leica cryostat (CM 1900). Alternatively, embryos were sectioned in a Leica vibratome (VT 1000S). Immunostaining was performed according to standard procedures.

Antibodies against the following proteins were used: green fluorescence protein (GFP) (Molecular Probes), anti-Myc (9E10, Santa Cruz), anti-HA (3F10, Roche), anti-Pax2 (Zymed), anti-Pax6 (CRP). Rabbit polyclonal antisera was used to detect Olig2 (Sun et al., 2001). Monoclonal antibodies to Foxa2 (4C7), Pax6, Pax7, Nkx2.2 (74.5A5), Islet1 (40.2D2), Lhx1/5 (4F2) were all obtained from the Developmental Studies Hybridoma Bank. Alexa488- and Alexa555-conjugated anti-mouse or anti-rabbit antibodies (Molecular Probes) were used. After single or double staining, sections were mounted, analysed and photographed using a Leica Confocal microscope.

Cell counting was carried out on 10-40 different sections of at least four different embryos after each experimental condition.

### In situ hybridization

Embryos were fixed overnight at 4°C in 4% paraformaldehyde in PB, rinsed and processed for whole-mount RNA in situ hybridization following standard procedures using probes for chick *WNT1*, *WNT3A*, *WNT4*, *TCF1*, *TCF3*, *TCF4*, *LEF1*, *GLI2*, *GLI3*, *DBX1*, *DBX2*, *NKX6.1*, *NKX6.2*, *BMP4*, *BMP7* and *noggin* (from the chicken EST project, UK-HGMP RC), or mouse *Gli2* and *Gli3* probes (Persson et al., 2002).

Hybridization was revealed by alkaline phosphatase-coupled anti-digoxigenin Fab fragments (Boehringer Mannheim). Hybridized embryos were postfixed in 4% paraformaldehyde, rinsed in PBT and vibratome sectioned.

### In vivo luciferase-reporter assay

Transcriptional activity assays of distinct components of the Shh/Gli and the  $\beta$ -catenin/Tcf pathways were performed in vivo. Chick embryos were electroporated at HH stage 11/12 with the indicated DNAs cloned into pCIG or with empty pCIG vector as control; together with a TOPFLASH luciferase reporter construct containing synthetic Tcf-binding sites (Korinek et al., 1998) and a renilla-luciferase reporter construct carrying the CMV immediate early enhancer promoter (Promega) for normalization. Alternatively transcriptional activity of indicated DNAs was also tested on a Gli-BS luciferase reporter construct containing synthetic Gli-binding sites (Sasaki et al., 1997).

Embryos were harvested after 24 hours incubation in ovo and GFP-positive neural tubes were dissected and homogenized with a douncer in Passive Lysis Buffer. Firefly- and renilla-luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega).

### Identification of HCNRs in the Gli3 locus and characterization of Tcfs binding sites

Sequence comparison of the Gli3 locus between different species was performed using the global alignment programme Shuffle-LAGAN (Brudno et al., 2003) and visualized with the VISTA visualization tool (Mayor et al., 2000). Human to Fugu Tcf/Lef conserved binding sites were found using rVISTA 2.0 searches for Tcf4 matrix from the TRANSFAC library.

Primers were designed to flank conserved sequences. PCRs were carried out using 100 pg of genomic chick DNA, and PCR-amplified fragments were transferred to the ptkEGFP expression vector (Uchikawa et al., 2003) for chick in ovo electroporation. Chick embryos were electroporated at HH stage 11/12 with each of the four identified HCNRs containing conserved Tcf-binding sites (HCNR1-4). Embryos were all co-electroporated with p-CMV-DsRed1 as electroporation control. Embryos were harvested 24 hours after electroporation, fixed 2-4 hours at 4°C in 4% paraformaldehyde in PB, rinsed and sectioned for GFP imaging on a Leica Confocal microscope.

Alternatively, PCR-amplified fragments were also transferred to the TKprom-pGL3-Basic vector carrying Luc+ (Promega) in which a TK minimal promoter was inserted. Embryos were electroporated with each of the four selected HCNRs (R1-R4) into TKprom-pGL3-Luc alone or together with  $\beta$ -catenin<sup>CA</sup> or Tcf<sup>DN</sup>, to check for their capacity to respond to Wnt activity. Embryos were all co-electroporated with a renilla-luciferase reporter construct for normalization, harvested after 24 hours incubation, and luciferase activity quantitated as above.

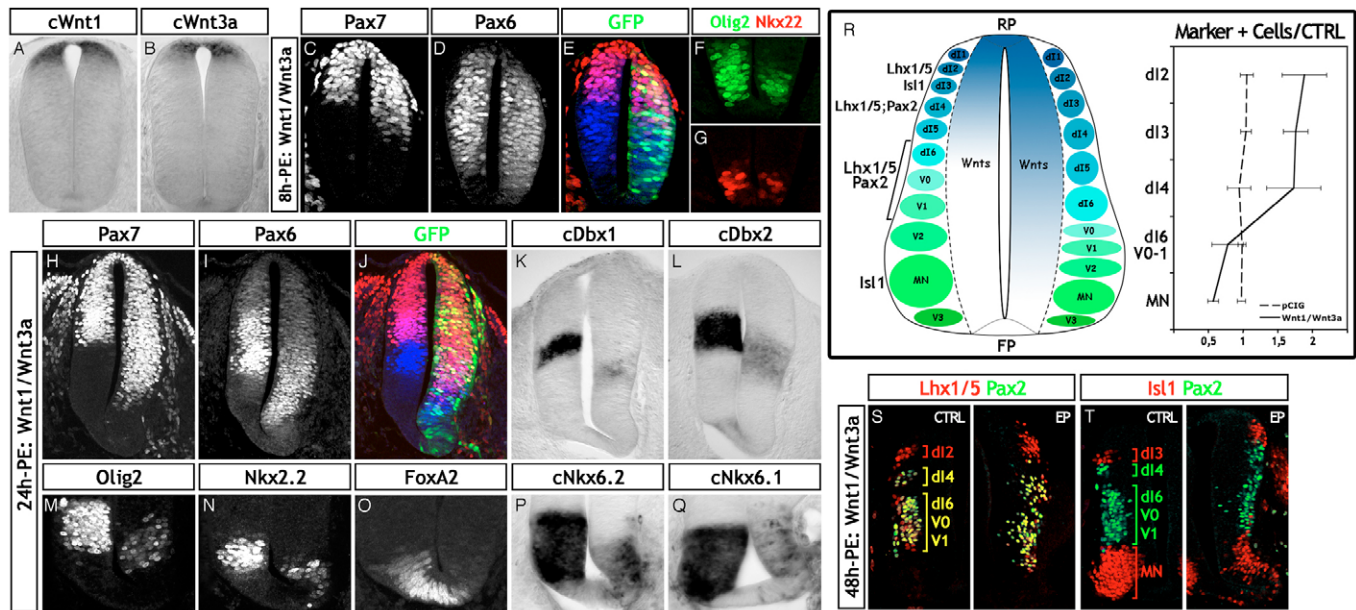
### Statistical analysis

Quantitative data were expressed as mean  $\pm$  s.d. or mean  $\pm$  s.e.m. Significant differences among groups were tested by Student's *t*-test.

## RESULTS

### Dorsally expressed Wnts pattern the neural tube through the canonical signalling

The Shh/Gli pathway plays a major role in DV pattern formation by activating expression of ventral class II progenitor proteins and repressing dorsal class I proteins. To begin to investigate an additional role for dorsally expressed Wnt genes in patterning the spinal cord, we misexpressed Wnt1/Wnt3a (Fig. 1A,B) along the DV axis, by in ovo electroporation, and analysed changes in the



**Fig. 1. Dorsally expressed Wnt genes regulate expression of progenitor proteins and result in cell fate changes along the DV axis.**

(A,B) HH stage 18 chick embryos show that expression of Wnt1 and Wnt3a is restricted to the dorsal-most neural tube. (C-G) Eight hours post-electroporation (8 hours PE) of Wnt1/3a, expression of Pax7 (C) and Pax6 (D) is ventrally expanded. Green shows GFP expression as reporter of ectopic Wnt gene expression (E). Expression of ventral Olig2 (F) and Nkx2.2 (G) is reduced. (H-Q) Twenty-four hours PE, Wnt1/3a causes overgrowth of the electroporated side, expansion of Pax7 (H) and Pax6 (I) expression, loss of intermediate genes Dbx1 (K) and Dbx2 (L), and loss of ventral Olig2 (M) and of Nkx2.2 (N), Foxa2 (O), Nkx6.2 (P) and Nkx6.1 (Q). (J) Green shows GFP expression as a reporter of ectopic Wnt expression. (R-T) Forty-eight hours PE, Wnt1/3a causes phenotype changes on differentiated neurons. (R) Quantitative analysis of *Lhx1/5+/Pax2-* dI2, *Islet1+* dI3 and *Lhx1/5+/Pax2+* dI4-dI6/V1 interneurons and of ventral *Islet1+* motoneurons (MN) on Wnt1/Wnt3a electroporated versus control pCIG electroporated embryos. (S,T) Double immunofluorescence staining with specific markers for each neuronal population: *Lhx1/5+/Pax2-* for dI2 (red); *Lhx1/5+/Pax2+* for dI4-dI6/V1 (yellow) (S) and *Islet1+* for dI3 and MN (red), Pax2 for dI4-V0/V1 (green) (T).

expression of several class II and class I genes as markers for progenitor domains. Phenotype analyses were all carried out at brachial level to avoid differences along the anteroposterior axis.

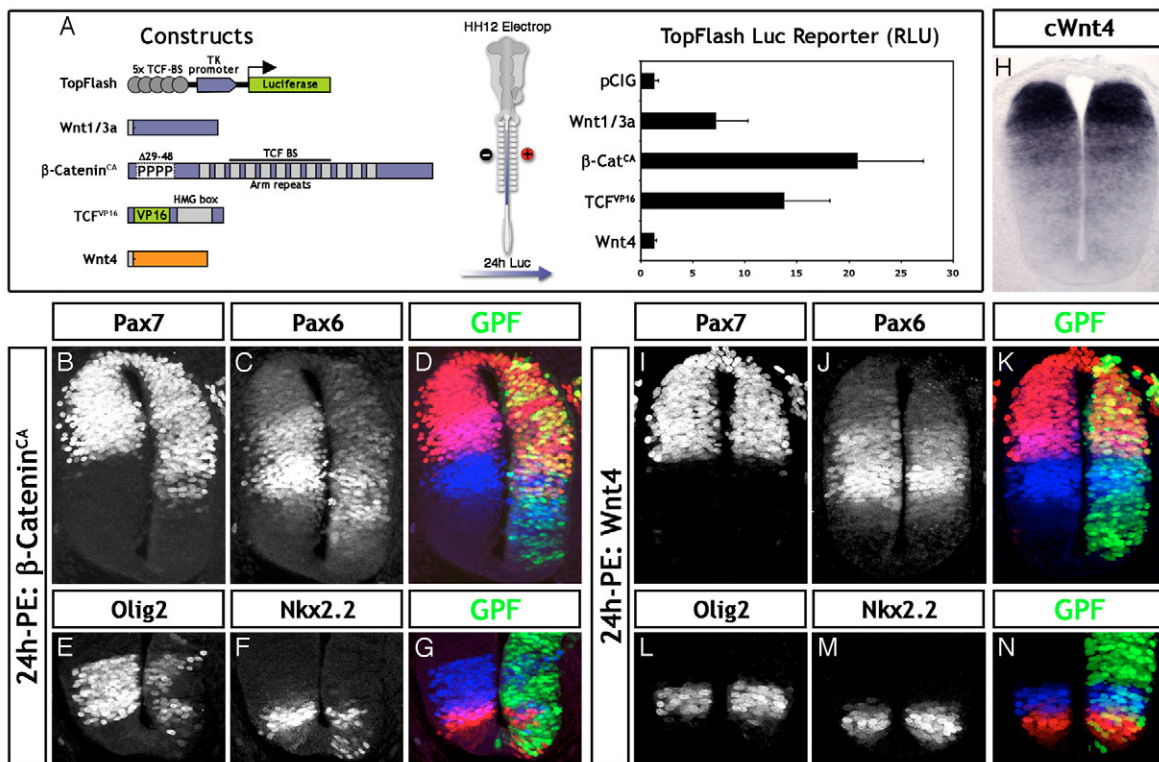
Stage HH11/12 chick embryos electroporated with Wnt1/Wnt3a and assayed 8 hours post-electroporation (PE) showed ectopic ventral activation of the dorsal genes Pax7 and Pax6 (Fig. 1C-E). Conversely expression of ventral genes such as Olig2 or Nkx2.2 was repressed (Fig. 1F,G). Twenty-four hours PE Wnt1/Wnt3a caused overgrowth of the neural tube, as previously reported (Megason and McMahon, 2002) (Fig. 1H-L). Additionally, expansion of the dorsal genes Pax7 and Pax6 was prominent (Fig. 1H-J), together with the loss of intermediate genes such as Dbx1 and Dbx2 (Fig. 1K,L) and the loss of ventral genes such as Olig2, Nkx2.2, Nkx6.1 and Nkx6.2 (Fig. 1M-Q). Furthermore, expression of the ventral-most floor-plate marker Foxa2, was repressed by Wnt activity (Fig. 1O).

During spinal cord development, six early born dorsal neuronal populations [dI1-6 interneurons (INs)] and five ventral neuronal populations [V3-V0 INs and motoneurons (MNs)] have been identified by the expression of HD factors and their position along the DV axis (Helms and Johnson, 2003; Jessell, 2000; Martí et al., 2005) (Fig. 1R).

To investigate whether changes in progenitor proteins resulted in phenotype changes in differentiated neurons, embryos electroporated with Wnt1/Wnt3a were assayed 48 hours PE for the expression of markers for specific neuronal populations (Fig. 1R). Overexpression of Wnt1/Wnt3a increased dorsal IN number (dI2, dI3 and dI4), at the expenses of the intermediate and the ventral IN subtypes dI6-V0/1 and MNs (Fig. 1R). This was detected as a 78.79% increase in *Lhx1/5+*; *Pax2-* dI2 neurons, as a 68.65%

increase in *Islet1+* dI3 neurons, and as a 83.35% increase in *Lhx1/5+*; *Pax2+* dI4 neurons. Conversely we detected a moderate (21.07%) decrease in *Lhx1/5+*; *Pax2+* dI6-V0/1 neurons, and a 41.58% decrease in *Islet1+* MNs (Fig. 1S,T). Thus, Wnt activity appears to regulate neuronal cell fate specification in a manner consistent with changes in progenitor proteins.

These results suggest a role for Wnt activity in DV patterning the spinal cord and raise the issue of whether this activity was mediated by the canonical Wnt pathway. Wnt signal is transduced through the canonical pathway to  $\beta$ -catenin, which then enters the nucleus and forms a complex with Tcfs (from T-cell factor) to activate transcription of Wnt target genes (Logan and Nusse, 2004). To begin to test the role of the canonical Wnt pathway, we first electroporated a stabilized form of  $\beta$ -catenin that is resistant to targeted proteolysis [and thus acts as a dominant active protein ( $\beta$ -catenin<sup>CA</sup>) (Tetsu and McCormick, 1999)].  $\beta$ -Catenin<sup>CA</sup> acts as a potent transcriptional activator on a TopFlash luciferase reporter assay containing synthetic Tcf-binding sites (Korinek et al., 1998) expressed in neural tube cells by in ovo electroporation (Fig. 2A). Embryos electroporated with  $\beta$ -catenin<sup>CA</sup> and assayed 24 hours PE, showed cell-autonomous ectopic activation of dorsal genes such as Pax7 or Pax6, together with a strong and cell-autonomous repression of ventral genes such as Olig2 or Nkx2.2 (Fig. 2B-G), a phenotype that was highly comparable with the electroporation of Wnt1/Wnt3a, and thus consistent with a role for the Wnt canonical pathway in DV patterning. Further indication for the canonical pathway in this role was the observation that electroporation of Wnt4, a Wnt gene expressed at high levels in the developing neural tube (Fig. 2H), but unable to activate Tcf-mediated transcription (Fig. 2A), resulted in



**Fig. 2. Wnt patterning activity is mediated by the canonical pathway.** (A) Activators of the canonical Wnt pathway (left) generate different transcriptional activities in vivo (centre). Embryos were electroporated with Wnt1/3a,  $\beta$ -catenin<sup>CA</sup>, Tcf<sup>VP16</sup> and Wnt4, together with the TopFlash reporter containing HMG-binding sites and normalization plasmid, and assayed 24 hours PE for luciferase activity. Graph shows normalized luciferase units. (B-G) Twenty-four hours PE,  $\beta$ -catenin<sup>CA</sup> caused expansion of Pax7 (B) and Pax6 (C) expression in those cells expressing GFP (D), and loss of ventral Olig2 (E) and Nkx2.2 (F) expression in a cell-autonomous way. (G) GFP expression on electroporated cells. (H) Expression of Wnt4 in a HH stage 18 embryo. (I-N) Twenty-four hours PE, Wnt4 produced wild-type expression of Pax7 (I) and Pax6 (J) in cells expressing GFP (K), and wild-type expression of Olig2 (L) and Nkx2.2 (M) in cells expressing GFP (N).

no changes on progenitor gene expression (Fig. 2I-N). These results prompted us to test a possible role for Tcf transcription factors in DV patterning.

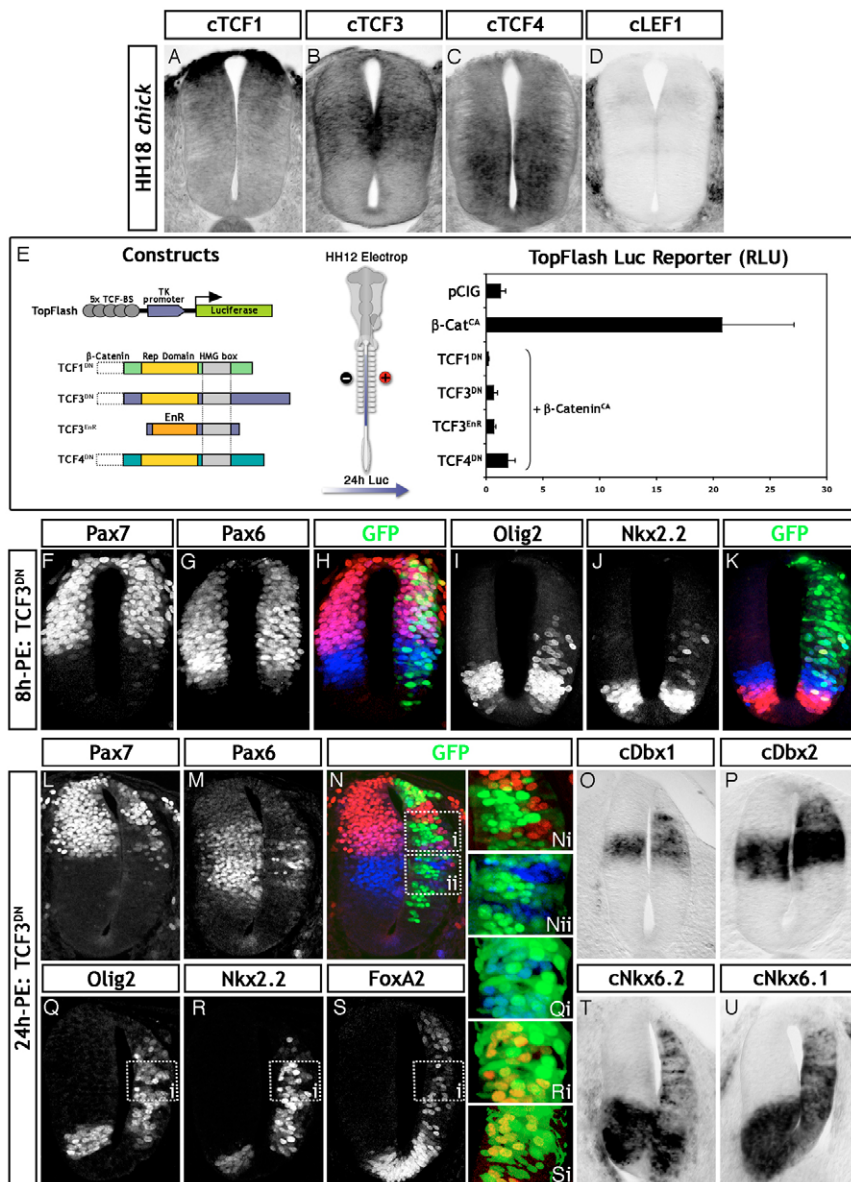
### Tcfs expressed throughout the DV axis regulate patterning of the neural tube

Activation of the canonical Wnt pathway results in Tcf target gene activation (Logan and Nusse, 2004). Members of the Tcf/Lef family of HMG-box transcription factors are differentially expressed in the developing spinal cord (Schmidt et al., 2004): Tcf1, Tcf3 and Tcf4 encompass the entire DV axis of the neural tube (Fig. 3A-D). To investigate a role for Tcf transcriptional activity along the DV axis, we electroporated dominant-negative (DN) forms of Tcf1, Tcf3 and Tcf4 that lack the  $\beta$ -catenin-interacting domain, thus acting as constitutive repressors of Wnt target genes (Kim et al., 2000; Tetsu and McCormick, 1999). Tcf1<sup>DN</sup>, Tcf3<sup>DN</sup> and Tcf4<sup>DN</sup> act as strong transcriptional repressors on the TopFlash luciferase reporter (Fig. 3E).

Eight hours PE of any Tcf<sup>DN</sup> was not sufficient time to induce changes in the expression of dorsal genes such as *Pax7* or *Pax6* (Fig. 3F-H); however, it did result in the rapid and cell-autonomous ectopic activation of ventral genes such as *Olig2* and *Nkx2.2* (Fig. 3I-K). This suggests that positive Wnt activity was required for the restriction of *Olig2* and *Nkx2.2* expression to their respective pMN and p3 progenitor domains.

Longer exposure to Tcf<sup>DN</sup> (24 hours PE), resulted in the strong and cell-autonomous repression of endogenous *Pax7* and *Pax6* expression (Fig. 3L-N), together with the dorsal expansion of intermediate genes such as *Dbx1* and *Dbx2* (Fig. 3O,P). Additionally, expression of ventral genes such as *Olig2*, *Nkx2.2*, *Nkx6.1* and *Nkx6.2*, as well as the ventral-most floor plate marker *Foxa2* were all ectopically activated (Fig. 3Q-U). Furthermore, electroporation of any of the three Tcf<sup>DN</sup> resulted in highly comparable ventralized neural tubes (see Fig. S1A-L in the supplementary material), with Tcf4<sup>DN</sup> being the weakest transcriptional repressor on the TopFlash reporter assay (Fig. 3E), and consistently the weakest de-repressor of ventral genes (see Fig. S1G-L in the supplementary material).

To further test whether these phenotype changes were due to repression of Tcf target genes, we took advantage of the HMG box DNA-binding domain of Tcf3 fused to the repressor domain of engrailed protein (Tcf3<sup>EnR</sup>) that acts as a strong transcriptional repressor on a luciferase assay in ovo (Fig. 3E). Electroporation of Tcf3<sup>EnR</sup> resulted in a phenotype identical to that of Tcf<sup>DN</sup> (see Fig. S1M-R in the supplementary material). Converse experiments electroporating the HMG box fused to the transactivator domain of VP16 (Tcf3<sup>VP16</sup>) resulted in the cell-autonomous loss of ventral gene expression (see Fig. S1S-U in the supplementary material), indicating that ventralization of the NT was indeed the consequence of Tcf-mediated transcriptional repression of target genes.



**Fig. 3. Tcfs expressed throughout the DV axis regulate patterning of the neural tube.**

(A-D) HH stage 18 embryos showing expression of chick TCF1 (A), chick TCF3 (B), chick TCF4 (C) and chick LEF1 (D). (E) In vivo quantitative analysis of the repressors activities of components of the canonical Wnt pathway. Embryos were electroporated with mutant versions of Tcf proteins. Diagram shows schematic representation of DNAs. Graphics show normalized luciferase units. All mutant Tcfs abolished  $\beta$ -catenin-induced luciferase activity. (F-K) Eight hours PE of Tcf3<sup>DN</sup>, dorsal Pax7 (F) and Pax6 (G) showed wild-type expression. GFP as a reporter of transgene expression (H). Ectopic expression of ventral Olig2 (I) and Nkx2.2 (J) was induced in cells expressing the transgene (K). (L-U) Twenty-four hours PE of Tcf3<sup>DN</sup>, expression of Pax7 (L) and Pax6 (M) was lost in electroporated cells. GFP as a reporter of transgene expression (N). Intermediate genes Dbx1 (O) and Dbx2 (P), and ventral genes Olig2 (Q), Nkx2.2 (R), Foxa2 (S), Nkx6.2 (T) and Nkx6.1 (U) were all dorsally activated.

Altogether, these results indicated an unexpected role for the Wnt canonical pathway in DV patterning, and prompted us to test for an interaction with other signalling pathways known to regulate patterning of the neural tube, such as the Bmp and the Shh/Gli pathways.

### Wnt patterning activity was independent of Bmp but dependent on Shh/Gli activity

It has long been proposed that a gradient of Bmps, secreted from roof plate cells, extends throughout the entire DV axis of the neural tube providing positional information and thus regulating pattern formation (Liu and Niswander, 2005). Additionally, Bmp activity regulates Wnts ligand expression in the dorsal neural tube indicating a genetic interaction of these pathways (Burstyn-Cohen et al., 2004; Chesnutt et al., 2004). Therefore, we first tested whether dorsalizing Wnt activity was dependent on Bmp activation.

Embryos electroporated with Wnt1/Wnt3a were assayed 24 hours PE for *Bmp4* and *Bmp7* expression by in situ hybridization. Results showed that expression of *Bmp4* was not modified, whereas *Bmp7* was ventrally expanded (Fig. 4A,B). We next

tested whether the Wnt1/3a dorsalizing activity was dependent on *Bmp7* by co-electroporation with the Bmp inhibitor noggin (Fig. 4C). At this developmental stage, inhibition of Bmp activity by overexpression of noggin had no effect on either dorsal (Pax7) or ventral (Olig2 and Nkx2.2) gene expression (Fig. 4D,E,G,H), which is related to previous report showing only minor changes in the dorsal-most dI1 after noggin misexpression (Fig. 4C) (Chesnutt et al., 2004). Co-electroporation of Wnt1/3a together with noggin showed that inhibition of Bmp activity had no effect on the Wnt1/3a-mediated expansion of Pax7 expression (Fig. 4F) or on the loss of ventral Nkx2.2 and Olig2 expression ( $23.3 \pm 16.1\%$  Nkx2.2+ cells and  $24.9 \pm 13.3\%$  Olig2+ cells 24 hours PE of Wnt1/Wnt3a;  $26.70 \pm 7.48$  Nkx2.2+ cells and  $23.45 \pm 5.18$  Olig2+ cells, 24 hours PE of Wnt1/Wnt3a together with noggin; Fig. 4I,S), thus indicating a role for the Wnt canonical pathway in DV patterning the spinal cord, independent of Bmp activity.

We next tested the hypothesis of a possible genetic interaction between the canonical Wnt/Tcf and the Shh/Gli pathways. Members of the Gli family of zinc-finger (ZnF) transcription

#### Fig. 4. Wnt activity in DV patterning is independent of Bmp but dependent on Shh/Gli activity.

(A,B) HH stage 11/12 embryos were electroporated with Wnt1 and Wnt3a, and analysed 24 hours PE, for the expression of Bmp4 and Bmp7 by in situ hybridization. (A) Bmp4 expression was weakly expanded ventral to the roof plate. (B) Ectopic Bmp7 expression was induced on the electroporated side.

(C) Electroporation of chick noggin resulted in the loss of Lhx2/9-expressing dl1.

(D,E) Noggin electroporation alone did not modify dorsal expression of Pax7. (F) Co-electroporation of Wnt1/3a with noggin resulted in the expansion of Pax7 expression.

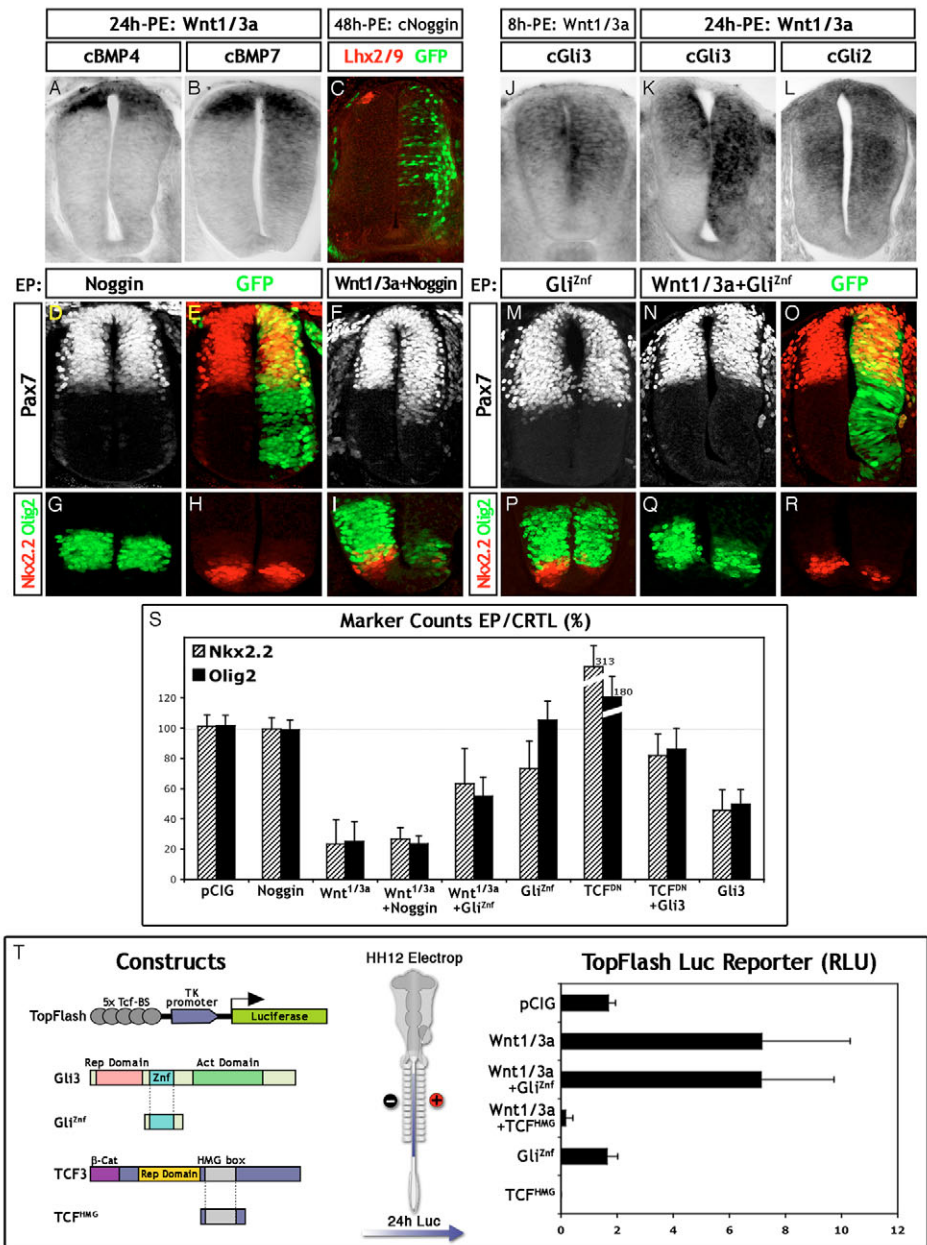
(G,H) Noggin electroporation alone resulted in the wild-type expression of Olig2 and Nkx2.2. (I) Co-electroporation of Wnt1/3a with noggin resulted in the reduction of Olig2 (green) and Nkx2.2 (red) expression.

(J-L) HH stage 11/12 embryos electroporated with Wnt1/3a were analysed 8 or 24 hours PE for the expression of *Gli3* and *Gli2* by in situ hybridization. (J) Eight hours PE of Wnt1/Wnt3a there was a moderate ventral expansion of *Gli3* expression. (K) Twenty-four hours PE, over- and ectopic expression of *Gli3* occurred. (L) Twenty-four hours PE there was overgrowth of the electroporated side without a change in expression of *Gli2*.

(M-R) Wnt regulation of progenitor gene expression is dependent on Gli activity. (M) Twenty-four hours PE of *Gli<sup>ZNF</sup>* alone there was no modification of Pax7 expression. (N,O) Co-electroporation of *Gli<sup>ZNF</sup>* abolished Wnt-induced ventral expansion of Pax7. (O) GFP as a reporter of transgene expression. (P) Twenty-four hours PE, *Gli<sup>ZNF</sup>* alone reduced Nkx2.2 (red) expression. (Q,R) Co-electroporation of Wnt1/3a and *Gli<sup>ZNF</sup>* resulted in a partial rescue of Olig2 (green) and Nkx2.2 (red) expression.

(S) Quantitative analysis of Olig2+ and Nkx2.2+ cells on electroporated versus non-electroporated side of the spinal cord (24 hours PE of indicated DNAs).

(T) In vivo quantitative analysis of the transcriptional activities for several components of the Wnt/ $\beta$ -catenin and the Shh/Gli pathways on a Tcf (TopFlash) transcriptional reporter. Embryos were electroporated with the indicated DNAs. Graph shows normalized luciferase units. *Gli<sup>ZNF</sup>* had no positive effect on the TopFlash reporter and cannot repress Wnt-induced luciferase. Electroporation of Tcf<sup>HMG</sup> blocked all induced luciferase activity.



factors are differentially expressed in the developing spinal cord and they appeared to have distinct transcriptional activities during NT pattern formation (Matisse and Joyner, 1999; Jacob and Briscoe, 2003). To assess for a possible genetic interaction between these pathways, we first tested whether Wnt activity regulated Gli expression. Embryos electroporated with Wnt1/Wnt3a, or with activator components of the pathway (data not shown), were assayed 8 and 24 hours PE for *Gli2* and *Gli3* expression by in situ hybridization. Ectopic Wnt1/3a expression caused a rapid (8h PE) and maintained (24 hours PE) ventral activation of *Gli3*, without affecting *Gli2* expression (Fig. 4J-L), suggesting that Wnt activity might be dependent on *Gli3* activation.

To test the hypothesis of a possible interaction between these pathways, we took advantage of a deleted form of *Gli3* protein that contained only the DNA binding zinc-finger-domain (*Gli<sup>ZNF</sup>*) (Cayuso et al., 2006). As expected *Gli<sup>ZNF</sup>* had no transcriptional activity on a TopFlash luciferase reporter containing only Tcf-binding sites (Fig. 4T), although it blocked Gli transcriptional activity on a Gli-BS luciferase assay (see Fig. S2A in the supplementary material). In vivo, electroporation of *Gli<sup>ZNF</sup>* had no effect on the expression of Pax7 (Fig. 4M) indicating that Gli activity was not required for the expression of Pax genes in the dorsal NT, as previously reported (Bai et al., 2004; Cayuso et al., 2006). However, in the ventral NT positive Gli activity was required for Nkx2.2 expression but not for Olig2 (Bai et al., 2004).

Consistently, blockade of all Gli-activities by electroporation of Gli<sup>ZNF</sup> precluded expression of Nkx2.2, resulting in only 73.2±18.3% Nkx2.2+ cells within p3 progenitor domain, although it was neutral on the expression of Olig2 (105±12.6% Olig2+ cells within pMN; Fig. 4P,S).

Co-electroporation of embryos with Wnt1/Wnt3a together with Gli<sup>ZNF</sup> showed that lack of Gli transcriptional activity completely abolished the Wnt induced ventral expansion of Pax7 and Pax6 (Fig. 4N,O; data not shown), suggesting that it was dependent on Gli repressor activity (Litington and Chiang, 2000; Stamatakis et al., 2005). Furthermore, co-electroporation of Gli<sup>ZNF</sup> together with Wnt1/Wnt3a resulted in a significant, although partial, rescue of ventral gene expression to 63.13±23.4% Nkx2.2+ cells and to 54.9±12.6% Olig2+ cells within their corresponding progenitor domains (Fig. 4Q-S). **This indicates that Wnt induced loss of ventral genes was, at least in part, mediated by Gli activity.**

Converse experiments were performed by co-electroporation of Tcf<sup>DN</sup> together with Gli<sup>ZNF</sup>. Results showed that repression of  $\beta$ -catenin/Tcf target genes resulted in the cell-autonomous loss of dorsal Pax7 and Pax6 expression and the ectopic activation of ventral genes, including Nkx2.2 and Olig2 (see Fig. S2B,G in the supplementary material). Indeed, electroporation of Tcf<sup>DN</sup> resulted in the remarkable increase to 313.0±41.6 Nkx2.2+ cells and 179.91±40.4 Olig2+ cells (Fig. 4S). Co-electroporation of Tcf<sup>DN</sup> together with Gli<sup>ZNF</sup> restored both dorsal Pax7 and ventral gene expression to 76.64±14.15 Nkx2.2+ and 117.45±20.91 Olig2+ cells (Fig. 4S) within their corresponding p3 and pMN progenitor domains (see Fig. S2C,D,H,I in the supplementary material). **Altogether, these data indicate that  $\beta$ -catenin/Tcf function in DV pattern is largely dependent on Gli activity.**

Therefore, **the induction of Gli3 may explain the inhibitory effect of Wnt on the ventral programme.** To this end, we tested the possibility that the overexpression of Gli3 was sufficient to mimic ectopic Wnt activation. Electroporation of full-length Gli3 resulted in a significant reduction of ventral gene expression (45.75±13.34 Nkx2.2+ and 49.70±9.53 Olig2+ cells, Fig. 4S) without inducing changes in dorsal gene expression (see Fig. S2L-Q in the supplementary material). Additionally we tested whether Gli3 was sufficient to rescue the ventralizing activity of Tcf<sup>DN</sup> by co-expression of Gli3 with the dominant negative Tcf. Gli3 electroporation reversed the ventralizing activity of Tcf<sup>DN</sup> [81.77±14.25 Nkx2.2+ and 85.99±13.67 Olig2+ cells (Fig. 4S) (see Fig. S2E,F,J,K in the supplementary material)].

### **Wnt signalling controls expression of Gli3 to restrict Shh/Gli activity**

Our results showing that Wnt activity was sufficient for the ectopic activation of Gli3 in the NT prompted us to test whether endogenous Wnt activity was required for Gli3 expression. Inhibition of Wnt transcriptional activity by electroporation of Tcf<sup>DN</sup>, resulted in the loss of Gli3 expression (Fig. 5A,B), without inducing changes on Gli2 expression (Fig. 5C). Furthermore, endogenous expression of Gli3 within the dorsal NT appeared to be dose dependent on Wnt activity, as mice mutant for Wnt1/3a (Muroyama et al., 2002) showed diminished Gli3 expression (Fig. 5D,E).

To explore the molecular mechanism by which the Wnt/ $\beta$ -catenin pathway regulated the expression of Gli3, we searched for highly conserved non-coding DNA regions (HCNR) within the human GLI3 locus that could work as potential enhancer modules. A total of 13 (R1-13) highly conserved non-coding DNA regions (HCNR) were found on a global alignment of ~300 kb, among widely divergent vertebrate species [including human, mouse, chick,

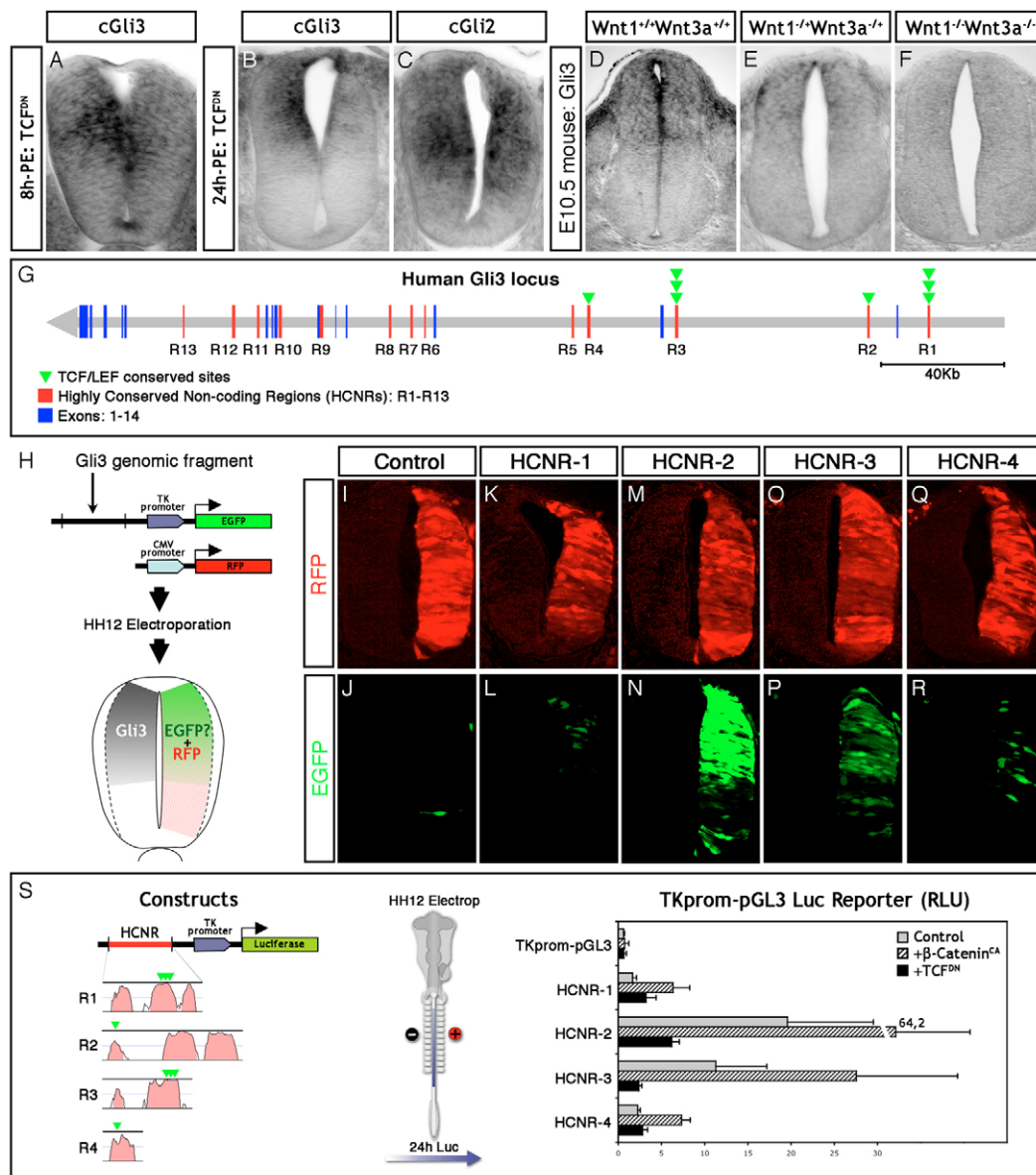
*Xenopus* and *Fugu* (see Fig. S3 in the supplementary material) (Abbasi et al., 2007)]. These HCNR are distributed across almost the entire GLI3 locus interval, with at least one element on each intron (Fig. 5G). The first four of these 13 HCNRs (R1-R4), located ~10 kb upstream of the GLI3 start codon (R1) and within the first and second introns (R2-R4) contained sequences that closely matched the core consensus Tcf/Lef1-binding sequence 5'-GTTTG-(A/T)(A/T)-3' (Van de Wetering et al., 1991) (see Fig. S3 in the supplementary material). Furthermore, two out of these highly conserved regions showed high density of potential Tcf-binding sites (R1 and R3) (Fig. 5G).

To assess the potential enhancer activity of these HCNR modules, selected genomic fragments R1-R4 were cloned into the ptk-EGFP expression vector (Uchikawa et al., 2003) for chick in ovo electroporation and monitored 24 hours PE for enhancer activity (Fig. 5H). Among the fragments tested, R2 and R3 directed GFP expression prominently in the dorsal NT, with R2 being particularly active, while R1 and R4 showed only very weak activity, although electroporation efficiently extended throughout the DV axis (Fig. 5I-R). These results indicate that R2 and R3 contain sufficient information to direct Gli3 expression to the dorsal NT.

In order to quantify the transcriptional activity of the Tcf/Lef1 conserved sites within the GLI3-HCNR1-4 modules, genomic fragments R1-R4 were cloned into TKprom-pGL3-Luc, which contains a minimal TK promoter upstream of a luciferase reporter gene. Embryos were electroporated with each of this GLI3-HCNR1 to R4 constructs alone or together with  $\beta$ -catenin<sup>CA</sup> or with Tcf<sup>DN</sup> in order to test for their responsiveness to  $\beta$ -catenin/Tcf transcriptional activity (Fig. 5S). Results showed that R2 and R3 alone were sufficient to activate reporter expression strongly (~19 and ~11 units respectively), whereas R1 and R4 showed moderate activity (~3 and ~7 units respectively), indicating that these HCNRs could be acting as enhancer modules in the neural tube by regulating Gli3 expression. Furthermore, co-electroporation with  $\beta$ -catenin<sup>CA</sup> increased R1-R4 transcriptional activity (R2 to ~64 and R3 to ~28 units), while co-electroporation with Tcf<sup>DN</sup> significantly reduced their activity (R2 to ~6 and R3 to ~2 units), indicating that these modules responded to Wnt/ $\beta$ -catenin and required Tcf activity for their strong activation (Fig. 5S).

Based on these results, we propose a model in which Wnt/Tcf signalling from the dorsal NT regulates the expression of the main inhibitor of the Shh/Gli pathway, Gli3. In turn, Gli3, acting mainly as a transcriptional repressor, restricts the graded Shh/Gli ventral activity. To test this hypothesis directly, we quantitatively assayed Gli and Tcf transcriptional activities on the Gli-BS luciferase reporter. Electroporation of Gli3<sup>Act</sup>, a strong activator of the Shh pathway (Stamatakis et al., 2005), resulted in ~9 units activation of the Gli-BS reporter; this activity was significantly reduced by co-electroporation with Wnt1/3a (Fig. 6A). Repression of Tcf transcriptional activity was sufficient for a ~4-fold transactivation of the Gli-BS reporter, suggesting that loss of Gli3 expression, and therefore loss of Gli3 mediated repression, was sufficient for Shh/Gli activation. In support of this, Tcf<sup>DN</sup> activation was lost by the co-electroporation of either Gli3 or by Tcf<sup>HMG</sup>, indicating an indirect transcriptional mechanism (Fig. 6A).

Altogether, our results show that the canonical Wnt/ $\beta$ -catenin pathway plays a pivotal role in the control of the graded activity of the Shh/Gli pathway. This control is largely achieved through the regulation of Gli3 expression, the main repressor of Shh/Gli activity (Fig. 6B).



**Fig. 5. Wnt signalling controls Gli3 expression in the dorsal spinal cord.** (A-C) Embryos were electroporated with Tcf<sup>DN</sup> and analysed for the expression of *Gli3* and *Gli2* by in situ hybridization. (A) Eight hours PE, Tcf<sup>DN</sup> caused a reduction of *Gli3*. (B) Twenty-four hours PE, co-electroporation of Tcf<sup>DN</sup> resulted in the loss of *Gli3* expression. (C) Twenty-four hours PE, co-electroporation of Tcf<sup>DN</sup> caused the reported growth arrest of the electroporated side, without changing expression of *Gli2*. (D-F) Analysis of mouse *Gli3* expression in wild-type (*Wnt1*<sup>+/+</sup>; *Wnt3a*<sup>+/+</sup>), double heterozygous (*Wnt1*<sup>+/-</sup>; *Wnt3a*<sup>+/-</sup>) and double homozygous (*Wnt1*<sup>-/-</sup>; *Wnt3a*<sup>-/-</sup>) 10.5 dpc mouse embryos, by in situ hybridization. (G) Schematic representation of the human *GLI3* locus. Conserved coding sequences are depicted in blue and conserved non-coding sequences in pink. Grey arrow indicates the length of the *GLI3* gene and the direction of transcription. Tcf-binding sites are depicted in green. (H-R) Activity of HCNR1-R4 as putative enhancers tested by in ovo electroporation. (H) Embryos were co-electroporated with pCMV-DsRed as electroporation control, and analysed 24 hours later for GFP and RFP expression. (I, J) Embryos electroporated with the control empty vector showed only red expression. (K, L) Embryos electroporated with R1 showed only weak dorsal GFP expression. (M, N) HCNR2 electroporation resulted in strong dorsal GFP expression. (O, P) HCNR3 electroporation resulted in dorsal GFP expression, although weaker. (Q, R) HCNR4 electroporation resulted in only weak GFP expression. (S) In vivo quantitative analysis of the transcriptional activities of HCNR1-4. Embryos were electroporated with each of the amplified HCNRs (R1-R4) alone, or together with β-catenin<sup>CA</sup> or Tcf<sup>DN</sup>. Embryos were all co-electroporated with a renilla-luciferase reporter construct for normalization, harvested after 24 hours of incubation and luciferase activity quantitated. Graph shows normalized luciferase units.

## DISCUSSION

Pattern formation is the process by which embryonic cells form ordered spatial arrangements in different tissues. In the developing spinal cord, DV patterning is achieved by the counteracting activities of morphogenetic signals secreted from dorsal and ventral signalling

centres. The Shh/Gli pathway plays a major role in patterning the ventral neural tube; however, the molecular mechanisms that limit target gene responses to specific progenitor domains remained unclear. In this study, we provide evidence that Wnts, by signalling through the canonical pathway, play an important role in DV





four enhancer modules within the human *GLI3* locus (Abbasi et al., 2007) in which core-consensus Tcf-binding sites are highly conserved throughout vertebrate species. We showed that two of these enhancer modules (HCNR2 and HCNR3) contain sufficient information to direct expression of *Gli3* to the dorsal spinal cord, and that activity of these two modules is dependent on  $\beta$ -Catenin/Tcf transcriptional activity. Although HCNR2 contained only one Tcf-binding site, it appeared to be highly efficient at directing *Gli3* expression to the dorsal NT, suggesting that other transcription factors might contribute to its effect.

Together, these results indicate that *Gli3*, the main repressor of the Shh/*Gli* activity, might be a direct target of Wnt/ $\beta$ -catenin. In turn, expression of *Gli3* within the dorsal NT serve to restrict Shh activity, therefore the balance of Shh and Wnt activities would be crucial to pattern the spinal cord along its DV axis. Shh and Wnt signals exhibit opposing functions in partitioning the somites (Borycki et al., 2000) and the otic vesicle (Riccomagno et al., 2005) along their DV axis. It would be of interest therefore to test whether Wnt regulation of *Gli3* expression in these tissues might be a conserved mechanism for opposing Hh/Wnt activities. However, analysis of the mice mutant for *Gli3* (Persson et al., 2002), as well as our data overexpressing full-length *Gli3*, suggested additional roles for Wnt function in DV pattern, particularly in the regulation of dorsal gene expression. One possibility is that Wnt activity might not only regulate expression of *Gli3* but also the balance between full-length and processed *Gli3* (i.e. transcriptional activator versus repressor) through modifications to either the phosphorylation state and/or proteolytic processing of this protein.

Additionally, a recent *in silico* analysis reported that the *Olig2* and the *Nkx2.2* loci have conserved canonical *Gli* and Tcf regulatory sequences (Hallikas et al., 2006). Our results showed that expression of Tcf4 in the ventral NT and repression of Tcf targets cause rapid and cell-autonomous expansion of *Olig2* and *Nkx2.2* expression. Conversely, Lei et al. (Lei et al., 2006) have recently reported a requirement for positive Wnt and Shh signalling for *Nkx2.2* expression. Although these data indicate that expression of ventral progenitor genes such as *Olig2* and *Nkx2.2*, in their correct cell numbers and within their appropriate progenitor domains, requires integration of signalling from both the canonical Wnt/Tcf and the Shh/*Gli* pathways, whether integration of these signals results in a cooperative or antagonistic transcriptional response remains a matter of controversy. Understanding the precise molecular mechanism for integration of these activities requires further experiments, although the recently reported direct interaction between *Gli* and  $\beta$ -catenin provides an attractive working model (Ulloa et al., 2007).

In addition, prominent signalling molecules resident in the roof plate are members of the Tgf $\beta$ /Bmp family, and it has long been proposed that a gradient of these proteins as they are secreted from roof-plate cells extends throughout the entire DV axis of the neural tube and regulates pattern formation (Liu and Niswander, 2005). Our results show that Wnt activity regulates Bmp expression; others have shown that Bmp activity regulate Wnt ligand expression in the dorsal neural tube (Burstyn-Cohen et al., 2004; Chesnutt et al., 2004), indicating a genetic interaction between these pathways. However, our results show Wnt-mediated regulation of dorsal gene expression to be independent of Bmp activity and we have recently reported a prominent role played by the Tgf $\beta$ /activin pathway in promoting cell cycle exit and neurogenesis, and in promoting differentiation of selected neuronal subtypes at the expense of other subtypes (García-Campmany and Martí, 2007). Together, these data suggest a model in which both pathways regulate cell fate specification and the balance between proliferation and

differentiation in a coordinate way. On the one hand, Wnts maintain progenitor cells cycling and restrict Shh/*Gli* graded activity to ensure the generation of the required amount of progenitor populations at their precise spatial DV locations. On the other hand, Tgf $\beta$ /Bmps promote cell cycle exit and the generation of specific neuronal subtypes, probably by coordinating pattern formation and neurogenesis. (Ille et al., 2007; Liu and Niswander, 2005).

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/2/237/DC1>

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