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"INTEGRATION OF SHH AND WNT PATHWAYS CONTROLS MORPHOGENESIS OF THE CNS"

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Barcelona, 4 de Abril de 2008

Divisió de Ciéncies Experimentals i Matemàtiques
Programa de Doctorat del Departament de Genètica
Facultat de Biologia
Universitat de Barcelona
Bienni 2003-2005

"INTEGRATION OF *SHH* AND *WNT* PATHWAYS CONTROLS MORPHOGENESIS OF THE CNS"

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RESUMEN

Relevancia de las vías de señalización celular

Las vías de señalización intercelular son elementos clave para la vida de cualquier organismo pluricelular. Durante el desarrollo embrionario, sólo unas pocas cascadas de señalización y sus respectivos ligandos aparecen reiterativamente en diferentes contextos para regular la comunicación célula-célula y para generar la información posicional necesaria para coordinar las actividades de un vasto número de células (Pires-daSilva y Sommer, 2003). Durante la vida adulta, los mismos mecanismos de comunicación celular son empleados para el mantenimiento de la homeostasis tisular o la regeneración de ciertos tejidos. La regulación de estas vías de comunicación es clave; una hiper- o hipo-activación de alguna de estas vías de señalización puede causar efectos devastadores como defectos durante el desarrollo embrionario o, más adelante, distintas malformaciones ó cáncer. Los mecanismos moleculares que modulan las diferentes actividades de estas cascadas de señalización en un tiempo y espacio determinados representan una de las mayores y más complejas situaciones que necesitan ser entendidas con profundidad para poder trazar el camino hacia la cura de muchas patologías humanas.

La familia de proteínas Wnt y Hedgegog costituyen moléculas de señalización celular que participan en la regulación de muchos aspectos del desarrollo de los metazoos a través de cascadas de señalización intracelular que recién empiezan a ser entendidas. Trabajos recientes evidencian que ambas vías de señalización tienen varios aspectos en común (Kalderón, 2002; Nusse, 2003).

Transducción de la señal de Wnt/ β-catenina

Un punto clave de la vía de señalización de los Wnt es la regulación de la estabilidad de la proteína citoplasmática β-catenina, la cual actúa como un co-activador transcripcional de la vía. En ausencia de la señal Wnt, un complejo citoplasmático formado por distintas proteínas de anclaje como Axin o APC, y kinasas, como Gsk3β ó CkIα, promueve la fosforilación de β-catenina que de esta manera queda marcada para su degradación a través del proteasoma. En el núcleo, la expresión de los genes diana de la vía es reprimida por la familia de factores de transcripción TCF/LEF1. Cuando un ligando Wnt se une al complejo receptor formado por Frizzled/ LRP, la señal es transducida intracelularmente por la proteíana citoplasmática Dishevelled que, a través de un mecanismo desconocido, bloquea la actividad del complejo citoplasmático de degradación. En estas condiciones β-catenina se acumula y transloca al núcleo, donde interacciona con TCF/LEF1 y activa la expresión de los genes diana (Logan y Nusse, 2004)

Transducción de la señal de Shh

La vía de Hh comparte varias características con la vía de Wnt. La regulación de un activador de la transcripción, Ci/Gli es crítica para la correcta señalización. En ausencia de Hh, la forma completa de Ci/Gli es parcialmente degradada, dejando el fragmento N-terminal de la proteína intacta. Esta forma se transloca al núcleo, donde recluta varios correpresores y bloquea la transcripción de genes diana de Hh. La degradación parcial de Ci/Gli es promovida por fosforilación a través de un complejo citoplasmático que también contiene proteínas de anclaje, como Cos2, y kinasas, como Gsk3β o Fused. La señal de Hh es transducida por otro complejo receptor constituido por Patched (Ptc) y Smoothened (Smo). Cuando Hh se une al receptor Ptc, éste libera la actividad de Smo que se acumula en la superficie celular e interactúa con Cos2. Esta interacción provoca la inhibición de la degradación de Gli/Ci y entonces la forma no procesada de la proteína se transloca activamente al núcleo, donde activa la expresión de genes diana (Ingham y McMahon, 2001).

Papel de la vía canónica de Wnt y Shh en la morphogénesis del tubo neural

Estas dos familias de proteínas de secreción pueden actuar sobre células vecinas como señales de corto alcance pero también como morfógenos, induciendo diferentes respuestas sobre células situadas lejos de

la fuente de producción en función de la concentración (Lander, 2007). Además, son varios los procesos durante el desarrollo que están simultáneamente regulados por las proteínas de señalización Wnt and Hh. En vertebrados uno de los sistemas donde estas dos vías de señalización convergen para controlar la especificación y la proliferación celular es el desarrollo de la médula espinal. Sin embargo, aun no se ha descrito un modelo integrativo que pueda relacionar las actividades de las señales Wnt y Hh en este contexto. Por esta razón, este trabajo está enfocado a la búsqueda y caracterización molecular de posibles interacciones entre estas dos importantes vías de señalización en la coordinación de la especificación y la proliferación de los precursores neuronales.

La actividad de proteínas de señalización, tales como Shh, Wnts o BMPs, regula la expresión y distribución de los dominios de expresión de genes de patrón como Pax6, Pax7, Olig2 o Nkx2.2. El perfil de expresión de estos factores de transcripción determina, para cada precursor, la especificación en los distintos tipos de neuronas que se originan a lo largo del eje dorsoventral (Gomez-Skarmeta et al., 2003). En el tubo neural, Shh, es secretado desde la notocorda primero y la placa del suelo después, y regula la expresión de genes de patrón a lo largo del eje dorsoventral. De esta manera se ha demostrado que Shh es suficiente y necesario para la generación de los diferentes subtipos de neuronas ventrales. En la parte dorsal del tubo neural, se ha propuesto que un gradiente de proteínas de señalización de la familia de las BMPs (*Bone Morphogenetic Proteins*), expresadas desde el ectodermo y la placa del techo, se extiende a lo largo del eje dorsoventral suministrando la información posicional necesaria para la generación de los diferentes tipos neuronales que se forman en regiones dorsales e intermedias (Nguyen et al., 2000). Además, también secretados desde la placa del techo, se ha propuesto que los ligandos Wnt1 y Wnt3a son necesarios para la formación de las poblaciones más dorsales del tubo neural (Muroyama et al., 2002), aunque una implicación directa en la regulación de genes de patrón no se ha descrito.

Paralelamente a la especificación de los diferentes subtipos neuronales, los progenitores proliferan en la zona ventricular del tubo neural para generar el número adecuado de neuronas de cada subtipo. El tamaño de cualquier órgano viene determinado principalmente por el número de células que lo constituyen, así como por el tamaño medio de las mismas. Número y tamaño celular están controlados y unidos por vías de señalización celular al los componentes de la maquinaria del ciclo celular (Murray, 2004). Existen suficientes evidencias experimentales como para asumir que las señales Wnt y Shh son fundamentales para regular la proliferación de los precursores neurales, y por tanto, el crecimiento del tubo neural (Cayuso y Martí, 2005). A nivel molecular, parece que ambas vías de señalización son capaces de regular la expresión de ciertos moduladores necesarios para la transición G1/S del ciclo celular como *ciclinaD1* o *N-myc* (Megason y McMahon, 2002, Cayuso et al., 2006).

A raíz del conjunto de estos conocimientos previos, surgen una serie de atractivas preguntas: i) ¿Están Wnts y Shh actuando en paralelo sobre diferentes precursores, por ejemplo, Wnts-dorsal y Shh-ventral? ii) ¿Están estas dos vías de señalización actuando sobre los mismos precursores, pero independientemente, regulando genes diana comunes? iii) ¿Existe una o más interacciones entre ambas vías a nivel de algún componente de la cascada de señalización intracelular? Para tratar de contestar a estas preguntas desarrollamos dos trabajos cuyo objetivo es la integración de estos dos tipos de señal en el contexto del control del crecimiento y la especificación celular a lo largo del eje dorsoventral del tubo neural del SNC en desarrollo.

Sistema modelo

El sistema modelo principal de elección para realizar este trabajo ha sido el embrión de pollo. Se trata de un sistema clásico dentro de la biología del desarrollo que ha contribuido a muchos de los más importantes avances dentro de este campo y de otros como la inmunología (Stern, 2005). Este modelo de vertebrado permite un cómodo acceso al embrión en cualquier estadio del desarrollo. Además, realizar experimentos de ganancia y pérdida de función in vivo

es relativamente fácil en muchos tejidos del organismo. El tubo neural representa, posiblemente, la parte del embrión donde la técnica de electroporación *in ovo* es más eficiente. A través de esta técnica se generan embriones mosaico que permiten el análisis de los resultados de una manera autónoma celular (Krull, 2004).

La vía Wnt/TCF controla la formación del patrón dorsoventral

Varias proteínas marcadoras de patrón se expresan a lo largo del eje dorsoventral del embrión en dominios parcialmente solapantes. Actuando en conjunto, bajo combinaciones específicas determinan la posición a lo largo del eje dorsoventral donde cada subtipo neuronal se diferencia. Nuestros resultados sugieren que Wnt1 y Wnt3a, a través de la vía canónica de Wnt, actúan como señales dorsalizantes en el control de la expresión de marcadores de progenitores neurales. De tal forma que la sobre-expresión de estos ligandos en el tubo neural causa la expansión de marcadores dorsales, tales como Pax6 o Pax7, a expensas de marcadores ventrales, como Olig2 o Nkx2.2. Además, demostramos que los cambios en la identidad de los progenitores neurales se mantienen en los subtipos de neuronas diferenciadas. Estos resultados son consistentes y complementarios al fenotipo presentado por los ratones doble knockout $Wnt1^{-/-}$; $Wnt3a^{-/-}$, en los que las poblaciones más dorsales del tubo neural están reducidas (Muroyama et al., 2002).

Por otro lado, experimentos de pérdida de función de la vía, para los cuales se usaron formas dominantes negativas de los distintos TCF expresados en el tubo (TCF-1,-3,-4) dieron como resultado una fuerte ventralización del tubo neural. Estos datos complementan los resultados obtenidos en los experimentos de ganancia de función y ponen de manifiesto el importante papel de la vía canónica de Wnt para la formación del patrón dorsoventral del tubo neural.

La regulación del patrón mediada por Wnts es independiente de BMPs

La actividad de BMPs parece ser fundamental para un adecuado establecimiento del patrón en las regiones más dorsales del tubo neural (Liu y Niswander, 2005). Nuestros resultados muestran que la actividad Wnt regula la expresión de BMP7. Para averiguar si la dorsalización causada por Wnts estaba mediada por la activación transcripcional de BMP7, co-expresamos el inhibidor extracelular de BMPs, Noggin, junto con Wnt1 y Wnt3a. Bajo estas condiciones, la activación de la vía de Wnt resultó en una dorsalización del tubo neural, indistinguible de la causada por Wnt1 y Wnt3a en ausencia de Noggin. Por lo tanto, al menos a nivel de ligando, el control que la vía de Wnt ejerce sobre la formación del patrón podría ser independiente de BMPs. Sin embargo, no podemos descartar que la vía canónica de Wnt esté regulando la vía de BMPs a otro nivel de la cascada de señalización.

La actividad Shh/Gli media el control de Wnts sobre el establecimiento del patrón dorsoventral

Existen tres miembros de la familia de factores de transcripción con dedos de zinc Gli en mamíferos y aves (Gli1-3). En el tubo neural, la combinación de los patrones de expresión de estos genes, junto con el gradiente de actividad de Shh, dan lugar a un gradiente de actividad Gli, en el cual Gli3 actúa como represor en regiones dorsales mientras que Gli1 y Gli2 lo hacen como activadores en regiones ventrales (Jacob y Briscoe, 2003). Nuestros resultados muestran que la activación de la vía canónica de Wnt incrementa los niveles de expresión de Gli3 sin alterar la actividad de Gli2. Además, cuando sobre-expresamos los ligandos Wn1 y Wnt3a en presencia de una forma truncada de Gli3, que sólo mantiene el dominio con los dedos de zinc necesarios para la unión al DNA y que no tiene dominios de transactivación, el fenotipo de dorsalización causado por Wnts es fuertemente reducido. Este resultado sugiere que, al menos en parte, la actividad dorsalizante de Wnts está mediada por la vía de Shh/Gli.

Wnt/β-catenina regula directamente la expresión de Gli3

Para caracterizar la regulación de Gli3 mediada por Wnts, realizamos experimentos de pérdida de función usando formas dominante-negativas de TCF1-4 y ratones knockout Wnt1$^{-/-}$; Wnt3a$^{-/-}$. En todos los casos, los niveles de expresión de Gli3 bajaron con la pérdida de función de la vía de Wnt. Estos resultados indican que la expresión dorsal de Gli3 en el tubo neural depende de la actividad Wnt.

Adicionalmente, realizamos un estudio *in silico* del locus humano de *Gli3* en busca de posibles regiones reguladoras conservadas que pudieran contener sitios consenso de unión al DNA para los factores de transcripción TCF/LEF1. Nuestros resultados demuestran la presencia de cuatro regiones con estas características, dos de las cuales recapitulan el patrón de expresión de Gli3 *in vivo* y responden a la actividad β-catenina/TCF en ensayos cuantitativos.

La vía canónica de Wnt controla la proliferación de precursores neurales

Señales de la vía de Wnt regulan la progresión a través del ciclo celular en diferentes áreas del SNC (Cayuso y Martí, 2005), incluido el tubo neural, el modelo aceptado actualmente sostiene que un gradiente de actividad Wnt1 y Wnt3a promueve la proliferación de los progenitores neurales (Megason y McMahon, 2002). Sin embargo, dado que estos ligandos sólo se expresan en la placa del techo, resulta difícil integrar este modelo en un tejido donde la tasa de proliferación se mantiene constante a lo largo del eje dorsoventral. Nuestros resultados muestran una actividad homogénea de la vía de Wnt a lo largo de todo el eje dorsoventral del tubo neural.

Por otro lado, el análisis de la distribución de las distintas fases del ciclo celular a través de citometría de flujo muestra que el control sobre el ciclo celular recae sobre la transición G1/S. Este resultado es consistente con la regulación directa que la vía realiza sobre ciclinaD1 (Shtutman et al., 1999; Megason y McMahon 2002).

Se requiere la actividad Shh/Gli para el control del ciclo celular y la expresión de ciclinaD1 mediados por Wnts

Además de la función de Wnts en el control de la proliferación en el tubo neural, Shh también regula este proceso en otras regiones del SNC (Cayuso y Martí, 2005), así como en el tubo neural (Cayuso et al., 2006). Por tanto, para buscar de qué manera ambas vías pueden regular simultáneamente la proliferación de los precursores neurales realizamos varios experimentos epistáticos. Los resultados obtenidos muestran un requerimiento de Shh para el control que Wnt ejerce sobre el ciclo celular. Además, estos experimentos ponen de manifiesto que este requerimiento depende de una interacción transcripcional entre ambas vías y sugieren que esta interacción pude de ser a nivel del control de la ciclinaD1.

Por otra parte, la sobre-expresión de ciclinaD1 bajo condiciones de pérdida de función de las vías de Wnt y Shh ponen de manifiesto que el control que la vía de Shh ejerce sobre la proliferación se extiende a la transición G2/S, mientras que la vía de Wnt tiene un control restringido a G1/S. Este resultado es consistente con trabajos recientemente publicados que proponen la regulación mediada por Shh/Gli de varios componentes del ciclo celular en G2 (Bénazéraf et al., 2006; Schüller et al., 2007; Locker et al., 2007)

INTRODUCTION

The Wnts and Hedgehogs families: history, evolution and biological significance

The similarities between the Wnt and Hedgegog (Hh) signalling commence from their respective discoveries. *Drosophila wingless* (*wg*) and *hh*, the first members of these protein families were identified together in the Nobel award winning genetic screen performed by Christiane Nüsslein-Volhard and Eric Wieschaus in 1980. Since that moment, both signalling molecules have been shown to be necessary for the development of many tissues and organs, and mutations in several components of their signalling cascades are implicated in degenerative diseases and cancer. Moreover, these pathways have been largely conserved during metazoan evolution, revealing a key role during development of all multicellular organisms.

As mentioned above, *wg* and *hh* were first identified in a screen for mutations disrupting the patterning of the *Drosophila* larva (Nusslein-Volhard and Weischhaus, 1980). Each thoracic and abdominal segment of the *Drosophila* embryo contains an anterior denticle belt, and a more posterior region of naked cuticle. In the *wg* mutant, the naked cuticle is absent, replaced by a disordered array of denticles. In a similar phenotype to that of *wg*, segmentation of the epidermis in *hh* mutant fly embryos is severely impaired as evidence by the formation of a denticle lawn projecting from the overlying ventral cuticle, that suggest the spines of a “hedgehog” to the discoverers. Posterior studies showed that *wg* activates the expression of the homeodomain-containing transcription factor *engrailed* (*en*) in adjacent narrow stripes of cells that form the border of each parasegment. At the same time, *en* activates the expression of *hh* that serve as a short-range signal to maintain *wg* expression, thus keeping an interdependent expression of Wg, En and Hh that is necessary for the establishment of segment polarity (Martinez Arias, 1989) (Figure 1).

The intervening years have shown that both signals have a broader role in Drosophila development, working as central patterning signals in the wing, leg, or eye imaginal discs as well as in germ-cell migration, gonads, gut or tracheal systems (Bejsovec, 2006; Jia and Jiang, 2006).

In 1982 Nusse and Varmus identified the mouse *int1* gene, as a preferential integration site for the Mouse Mammary Tumor Virus in virally induced breast tumors (Nusse and Varmus, 1982). When sequenced, the *int1* proto-oncogene was seen to encode a secreted protein that is cysteine rich. Several years later, *Drosophila wg* was shown to be a fly homolog of *int1* (Rijsewijk et al., 1987) and was renamed Wnt1, from “wingless” and “int1”, and hence the origin of the current name of this protein family.

In 1989, McMahon and Moon (McMahon and Moon, 1989) observed a duplication of the body axis in *Xenopus* following injection of mouse *wnt1* mRNA into ventral blastomeres of embryos at the 4-cell stage. This observation supported the notion that Wnt signalling was shared between vertebrates and invertebrates and, moreover, provided a rapid and convenient assay to study components of the Wnt pathway in vertebrates.

The cloning of *hh* was eagerly awaited and in 1992, three different groups show that *hh* encodes a putative secreted peptide whose expression was confined to different tissues in the *Drosophila* embryo (Lee et al.; Mohler and Vani; Tabata et al.; 1992). Vertebrate *hedgehog* genes were first reported in 1993 following a collaborative effort involving three groups (Echeland et al.; Krauss et al.; Riddle et al.; 1993). Unlike the fly, which has a single *hh* gene, there are several related genes in vertebrate species.

The combined observations made in vertebrates and invertebrates, together with genome sequencing, delineated Wnt and Hh as highly conserved signalling pathways. In mammals there are 19 secreted Wnt proteins, which can be divided into 12 conserved Wnt subfamilies. Of these, only 6 subfamilies have counterparts in ecdysozoan animals such as *Drosophila* and *Caenorhabditis* (Miller 2001). In contrast, at least 11 of the Wnt subfamilies occur in the genome of cnidarians. This finding suggests that some Wnt subfamilies were lost during the evolution of the ecdysozoan lineage (Kusserow et al., 2005).

As for the *wnt* family, *hh* genes are evolutionarily conserved. They have been identified in several invertebrate species including the leech, sea urchin

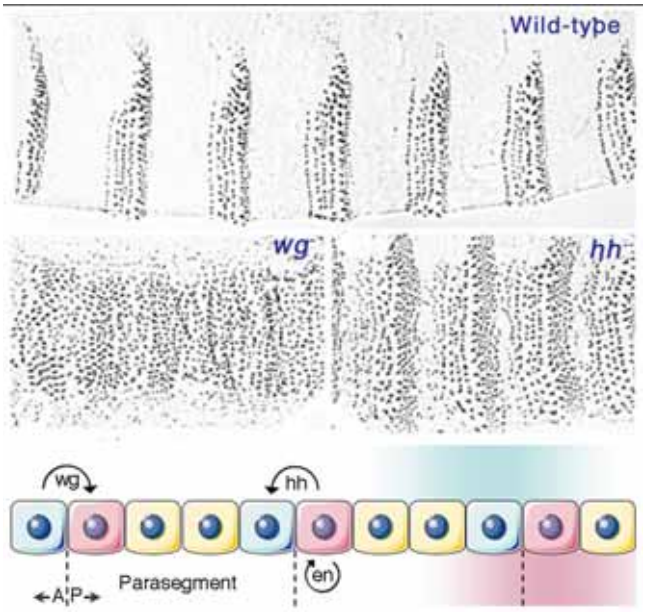

Figure 1. Wg and Hh signaling have essential roles in patterning the embryonic epidermis. In a wild-type embryo, as shown, the cuticle has an alternating pattern of naked cuticle and cuticle covered with small hairs called denticles. The wg and hh mutant embryos are devoid of naked cuticle. At early stages of embryonic development, wg acts over a short range posteriorly to maintain hh expression. Hh acts, in turn, anteriorly to maintain wg expression. The position of the compartmental boundary that separates each segment, called the parasegmental boundary, is marked by the juxtaposition of Wg- and Hh-expressing cells (shown in blue and red, respectively).

or the cephalochordate amphioxus (Pires-daSilva and Sommer, 2003). More recently, a transmembrane protein called Hedling that contains the N-terminal signalling domain of Hh has been shown to be present in sponges and cnidarians (Adamska et al., 2007). In mammals and birds, there are three homologues of the *Drosophila hh*: *Indian hedgegog* (*Ihh*), *Desert hedgegog* (*Dhh*) and *Sonic hedgehog* (*Shh*) (Echelard et al.; Krauss et al.; Riddle et al.; 1993). These represent three different subfamilies, some of which have been expanded by gene duplication in various other taxa. Thus, zebrafish has two *Shh* subfamily members, *twhh* and *shh* and two *Ihh* subfamily members, *qhh* and *ehh*. In addition, *Ihh* is given the name *bhh* in *Xenopus,* and *Xenopus* has two *Dhh* subfamily members, *hh4* and *chh*.

There are a few parts of the vertebrate body plan that are not influenced in some way by Hh or Wnt signalling (Ingham and McMahon, 2002; Logan and Nusse, 2004). Focused on the CNS, one classic example is the *wnt1/wnt3a* double knockout, which demonstrates a requirement for Wnt signalling in a wide region of the dorsal CNS (Ikeya et al., 1997). Of special interest is the expression of Shh in two key signalling centers in the vertebrate embryo: the notochord and the floor plate. Pioneer studies of neural plate patterning had shown the importance of notochord and floor plate signals in ventralizing the developing neural tube (Placzek, 1995). Ectopic expression experiments first (Echelard et al., 1993), and *Shh* knockout mice analysis later (Chiang et al., 1996), furnished the initial evidence that Shh regulates these processes.

In 1991 the *adenomatous polyposis coli* (*APC*) gene was discovered in a hereditary cancer syndrome termed familial adenomatous polyposis (FAP) (Kinzler et al., 1991; Nishisho et al., 1991). Two years later, the large cytoplasmic APC protein was found to interact with β-catenin (Rubinfeld et al., 1993; Su et al., 1993). This observation, together with the int1 gene discovery, bore out the connection between the Wnt pathway and human cancer. Mutations that promote constitutive activation of the Wnt signalling pathway lead to a variety of other tumor types (Giles et al., 2003), as well as other abnormalities such as tetra-amelia, bone density defects or tooth agenesis (Logan and Nusse, 2004). On the other hand, misregulation of Hh signalling have been shown to contribute to various pathologies, most notably different cancers including basal cell carcinoma, the most prevalent cancer in the Caucasian population, medulloblastoma or pancreatic adenocarcinoma (Ruiz i Altaba et al., 2002). Other important abnormalities include holoprosencephaly, a birth defect caused by a reduction of Shh, or polydactyly (Nieuwenhuis and Hui, 2004).

During the past two decades Wnt and Hh signalling pathways have seen implicated in a plethora of processes during development and adulthood. Comparative genomic analysis underscores the crucial role that *Wnt* and *Hh* genes play in embryonic patterning throughout the animal kingdom and reveals that a complex inventory of Wnt and Hh factors was present in multicellular animals well before the Cambrian explosion, 550 million years ago. Although twenty years have little to do with metazoans history, the current understanding opens the opportunity to start looking at possible interactions between both pathways that could explain how these signalling

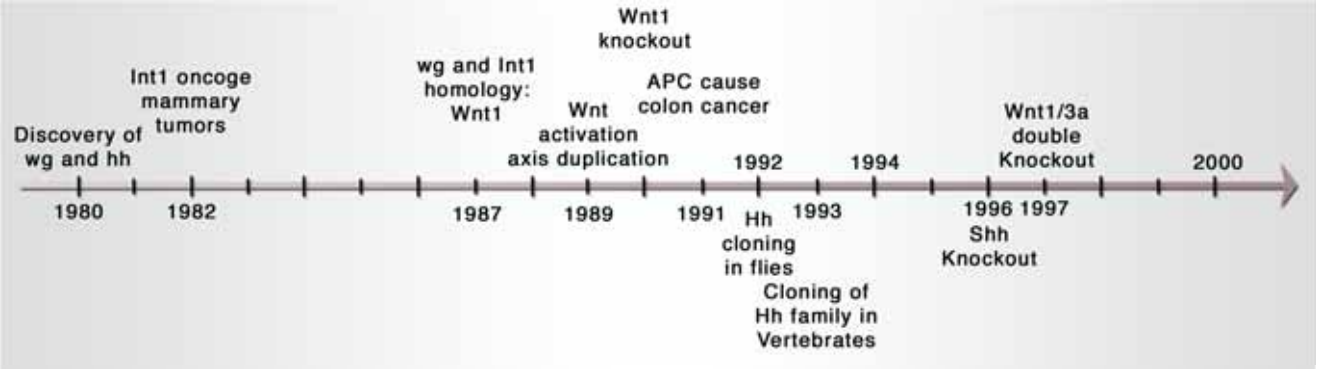

Figure 2. Chronologic diagram showing some major events during Wnts and Hh history.

molecules acting in common scenarios could control the same processes (Figure 2).

Wnts and Hh signalling

Wnt and Hh proteins: lipid-modified signals

Both the Wnt and the Hh proteins, which are not related in sequence to each other, are destined for secretion. Because molecules that are secreted from cells are commonly glycosylated but not, as far as known, acylated, the discovery that Wnt and Hh proteins carry covalently attached palmitates came as a surprise. Interestingly, the two acyltransferases, Porcupine (Porc) and Skinny-hedgehog (Skn), which have been respectively suggested to catalyse the addition of the acyl groups to Wnt and Hh proteins, are evolutionary related (Figure 3).

The biochemical nature of active Wnt proteins remained elusive until recently. At the primary sequence level, Wnt proteins share a nearly invariant pattern of 23 Cys residues (Miller, 2002). Some of these Cys residues probably form intramolecular disulphide bonds and are important for the proper folding of Wnt proteins. Although not evident from the primary sequence, recent works have shown that at least two lipid modifications are present on the mature Wnt. The first, is a palmitate group added to the conserved Cys77 in murine Wnt3a (Willert et al., 2003). This residue and, by extrapolation, the palmitoylation seem to be conserved in all Wnt proteins (Zhai et al., 2004). The second lipid modification of mouse Wnt3a consists on an unsaturated fatty acid (palmitoleic acid) that is attached to another conserved residue, Ser209 in murine Wnt3a (Takada et al., 2006). Furthermore, Wnt proteins are, unlike Hedgehog proteins, usually glycosylated on conserved N-linked glycosylation sites (Mason et al., 1992).

Porcupine (Porc) encodes a putative multipass transmembrane protein that is localized to the Endoplasmic Reticulum (Figure 3) and is conserved from invertebrates to mammals (Kadowaki et al., 1996; Tanaka et al., 2000). Wnt secretion and the numerous functions of *wg* in *Drosophila* development are abrogated in the absence of *porc* (Kadowaki et al., 1996). Moreover, Porc can bind to a domain in Wg that encompasses the acylation site (Tanaka et al., 2002). Recently, Porc has been shown to regulate the activity and distribution of Wnt proteins in the neural tube (Galli et al., 2007).

The processing of Hh is unusual in a number of aspects: Hh is made initially as a precursor molecule that consists of a C-terminal protease domain and an N-terminal signalling unit. The C-terminal protease of Hh cleaves the precursor in an autocatalytic manner to release the active signalling domain of Hh called Hh-Np (Lee et al., 1994). During this cleavage, the C-terminus of the Hh-Np form becomes covalently modified by the addition of a cholesterol molecule (Porter et al., 1996). After its signal sequence has been removed, the N-terminus of Hh becomes modified by the fatty acid palmitate on a conserved cysteine residue that is exposed at the very N-terminal end of the protein (Pepinsky et al., 1998) (Figure 3).

The *skinny-hedgegog* gene (Skn) encodes a transmembrane protein with homology to some

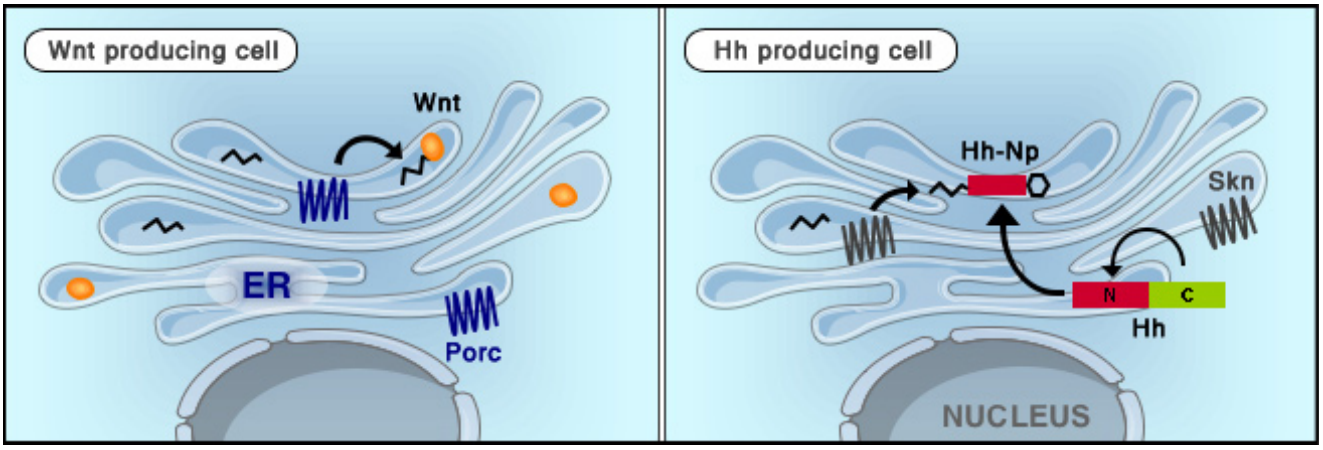

Figure 3. Production of lipid-modified Wnt and Hh proteins. Porcupine (Porc) is an endoplasmic reticulum (ER) protein that is required in Wnt producing cells, and which is thought to attach palmitate groups to Wnt. In Hh-producing cells, The C-terminus of Hh is cholesterol-modified during autocatalytic cleavage. Skinny-hedgehog (Skn) is necessary for the acylation of the Hh protein.

mammalian acyl transferases. Genetic analyses showed that *skn* is required in Hh-producing cells (Chamoun et al., 2001; Lee and Treisman, 2001). Cultured cells in which *skn* function is blocked by RNA interference secrete a form of Hh that lacks palmitate (Chamoun et al., 2001) (Figure 3).

The functions of all these modifications are not completely understood, but the reported data suggest that lipid-modifications together with glycosylation are necessary for correct intracellular targeting and secretion, and for extracellular spreading and full-activity of the ligand in the responding tissue. It is likely that Skn and Porc are indeed the enzymes that acylate Hh and Wnt proteins respectively, but direct biochemical evidence for this still lacking.

Trafficking, release and transport of Wnt and Hh proteins

Since Wnt and Hh proteins are covalently linked to lipids, they have high affinity for cell membranes. Despite this, the proteins are released and can act on cells distant from the source of production. Wnt and Hh families require the carrier molecules Wntless (Wls) and Dispatched (Disp), which bind and transport them to the membrane for releasing. Moreover, heparan sulfate proteoglycans stabilize Wnt and Hh and disperse them throughout tissues whereas lipoprotein particles are required for long-range signalling (Figure 4).

Wnt secretion appears to be tightly regulated by the highly conserved seven-pass transmembrane protein Wntless/Evi (Wls) that is a G-protein-couple receptor like the Wnt-signalling receptors, Frizzled proteins (Wistrand et al., 2006). Wls resides primarly in the Golgi apparatus (Figure 4), where it colocalizes and physically interacts with Wnts (Banziger et al., 2006; Bartscherer et al., 2006). In the absence of Wls, Wnt is retained within its producing cells, resulting in a Wnt loss-of-function phenotype. Moreover, the Retromer Complex, a macromolecular protein complex involved in intracellular trafficking (Figure 4), have been recently shown to sustained a Wls traffic loop from the Golgi to the plasma membrane and back to the Golgi, thereby enabling Wls to direct Wnt secretion (Port et al., 2008; Franch-Marro et al., 2008).

The release of Hh from cells requires a dedicated transport molecule: a protein called Dispatched (Disp). Initially found in *Drosophila* (Burke et al., 1999) but functionally conserved in mammals (Caspary et al., 2002; Kawakami et al., 2002), Disp is a multiple-pass transmembrane protein (Figure 4). In the absence of Disp, Hh is not secreted from cells and is unable to signal to neighbouring cells. Disp is required specifically for Hh signalling and is thought to contain a sterol-sensing domain that might interact with the cholesterol modification of Hh to facilitate its release from the membrane. Interestingly, the Hh receptor, Patched, and Disp are structurally related (Burke et al., 1999).

Heparan-sulfated forms of proteoglycans (HSPG) are long proteins with branched sugar side chains

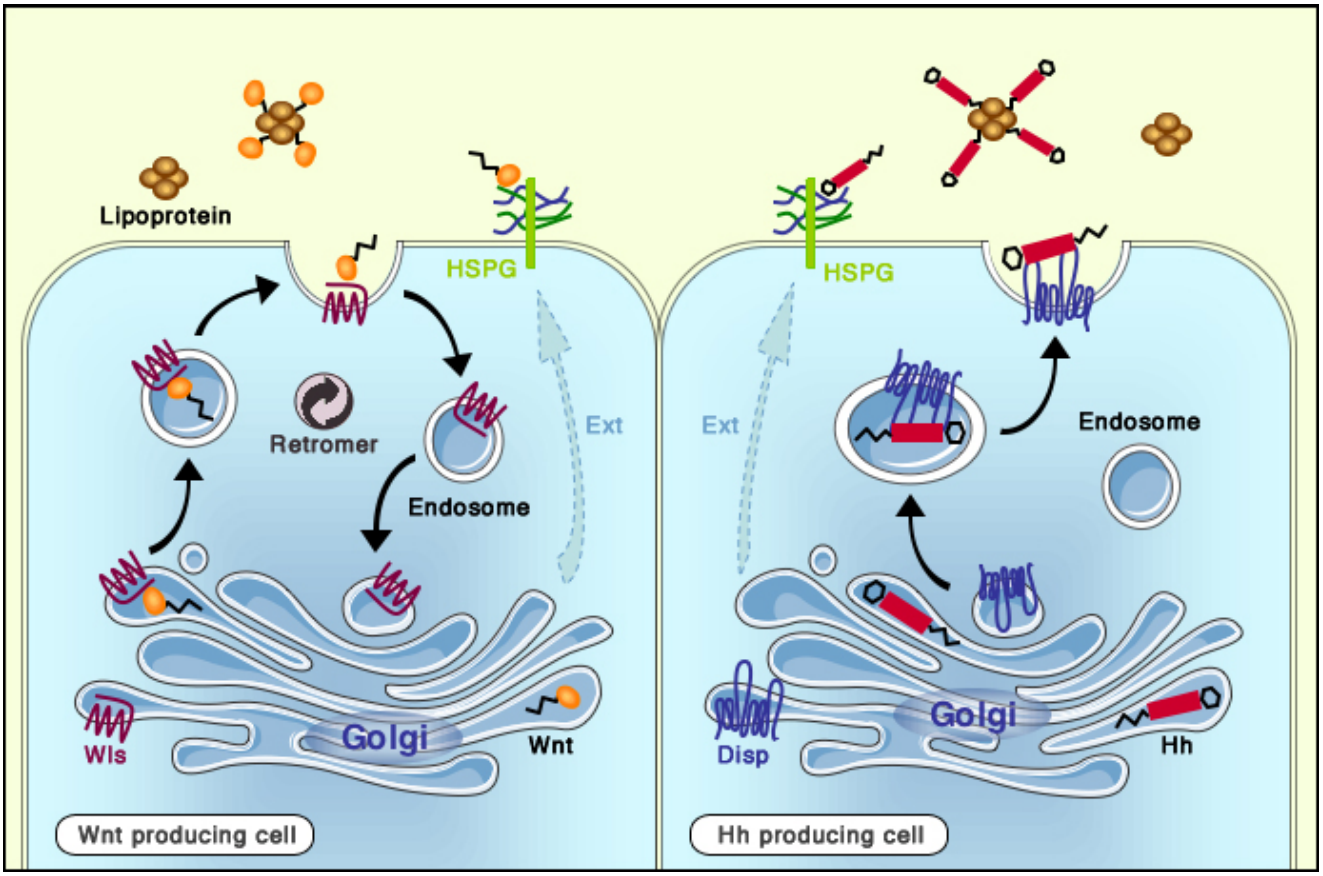

Figure 4. Intracellular trafficking, release and transport of Wnt and Hh proteins. In Wnt producing cells, transport and secretion of the Wnt protein in secretory vesicles is controlled by the multipass transmembrane protein Wntless (Wls) which is present in the Golgi and/or plasma membrane. The retromer complex regulates a Wls traffic loop between the Golgi and the plasma membrane. In Hh producing cells, intracellular trafficking and release of Hh requires the transmembrane protein Dispatched (Disp). EXT family members of glycosyltransferases and Heparin-sulfated forms of proteoglycans (HSPG) are also involved in Wnt and Hh extracellular diffusion.

that are expressed on the cell surface (Nybakken and Perrimon, 2002). In Drosophila, mutations in any of the three EXT family members of glycosyltransferases (Ext), or in other enzymes involved in HS chain biosynthesis, cause defects in Wnt or Hh distribution and signalling (Lin, 2004). The HSPG core proteins Dally (Dly) and Dally-like (Dlp), which are EXT family substrates (Figure 4), have been shown to prevent the accumulation of Wg and Hh in both producing and receiving tissue (Baeg et al., 2001, Han et al., 2004). In humans *EXT1* and *EXT2* mutations cause hereditary multiple exostoses (HME), a benign bone tumor characterized by multiple cartilage-capped bone outgrowths (Zak et al., 2002). The expression patterns of *EXT1* and *EXT2* in mice are consistent with a role in mediating *Ihh* transport, which would be consistent with the phenotypes observed (Stickens *et al.,* 2000).

Lipoproteins particles consist of a phospholipid monolayer surrounding a core of esterified cholesterol and triglyceride, and they are scaffolded by one of a family of apolipoproteins. Biochemical studies in Drosophila have shown that both Wingless and Hedgehog associate with lipoproteins in vivo. Knockdown of Lipophorin, one of these apolipoproteins, shows that these particles are required for long-range, but not short-range, signalling activity of Wingless and Hedgehog (Panáková et al., 2005) (Figure 4).

The prediction that Wls and Disp are evolutionary related to Fz and Ptc receptors respectively, raised the idea of 'intracellular receptor-mediated exocytosis' a hopeful working field. Wnt and Hh proteins are thought to act on neighbouring cells, but also on more distant cells as morphogens. However, it is unclear how these short- and long-range signalling events are modulated. HSPG have been proposed to control these processes by keeping Wnt and Hh proteins at the cell surface by a direct interaction, by protecting them from extracellular proteinases-mediated degradation, or indirectly,

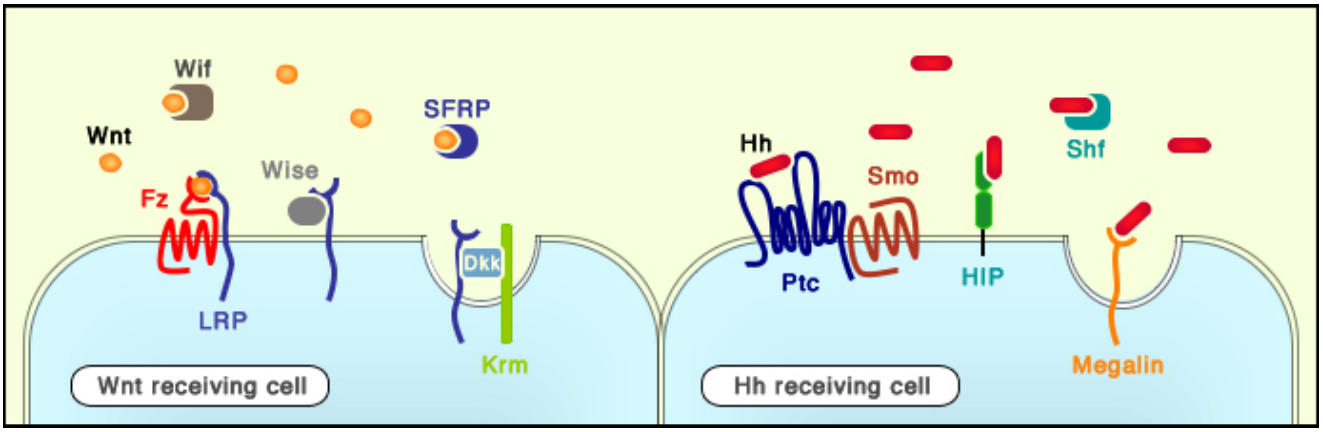

Figure 5. Cell surface and secreted molecules implicated in Wnt and Hh signaling. In vertebrates, Wnt proteins are inhibited by direct binding to either secreted Frizzled-related proteins (SFRP) or Wnt inhibitory factor-1 (Wif1). SFRP is similar in sequence to the cysteine-rich domain (CRD) of Frizzled, one of the Wnt receptors. The Wnt inhibitors Dickkopf (Dkk) and Wise bind to the Wnt co-receptors LDL receptor-related protein (LRP). Dkk also interacts with Kremen (Krm) to downregulate these molecules from the surface. Hh receiving cells requires the membrane receptors Smoothened (Smo) and Patched (Ptc). Megalin, a protein related to LRP has also been found to bind to Hh, but only in vertebrates. The Hedgehog inhibitory protein HIP binds to Hh and inhibits its function. Shifted (shf) is an extracellular inhibitor of Hh that is close related to Wnt inhibitor Wif.

by controlling the interaction with their respective receptors. Furthermore, the role of lipoprotein particles suggests that these morphogens could mediate short- and long-range signalling by different mechanisms.

Receptors and extracellular modulators

Cells employ multiple receptors to receive instructions from Wnt and Hh signals, in complex and little understood configurations. Genetic and biochemical data evidence that Frizzled (Fz) and Patched (Ptc) proteins are the primary receptors for Wnt and Hh ligands. Both receptors require the presence of other membrane partners, LRP and Smoothened (Smo) respectively, which are crucial for the modulation of the signal. Moreover, a number of extracellular and cell surface proteins, such as Wif-1, SFRP, Dkk or Wise on the Wnt pathway, and Shifted (Shf), Hedhehog Interacting Protein (HIP) or Megalin can on the Hh pathway, interact with the ligands or the receptors to modulate Wnt and Hh signalling (Figure 5).

Frizzleds (Fz) are seven transmembrane receptors with a long N-terminal extension called cysteine-rich-domain (CRD) (Figure 5). Wnt proteins binds directly to the Fz CRD and over-expression of Fz receptor fails to activate Wnt signalling unless its cognate ligand is present (Bhanot et al., 1996). In vertebrates, the Wnt-Fz interaction appears promiscuous, in that a single Wnt can bind multiple Frizzled proteins and viceversa (i.e. Bhanot et al., 1996). Moreover, Wnt and Wingless signalling also requires a single-pass transmembrane molecule of the low-density lipoprotein receptor family (LRP5/6), *arrow* in Drosophila (Wehrli et al., 2000; Pinson et al., 2000) (Figure 5). It has been proposed (Tamai et al., 2000), but not always confirmed (Wu and Nusse, 2002), that Wnt molecules can also bind to LRP and form a trimeric complex with a Frizzled.

Soluble Frizzled-Related Proteins (SFRP) resemble the ligand-binding CRD domain of the Frizzled family of Wnt receptors (Hoang et al., 1996) (Figure 5). SFRPs and Wifs are believed to function as extracellular Wnt inhibitors but, depending on context, may also promote signalling by Wnt stabilization or by facilitating Wnt secretion or transport (Logan and Nusse, 2004). As an alternative way of blocking Wnt signalling, Dickkopf-1 (Dkk) (Glinka et al., 1998) and the Wnt-modulator-in-surface-ectoderm (Wise) protein (Itasaki et al., 2003) modulate Wnt signalling by interacting with LRP5/6 (Figure 5). Dkk crosslinks LRP6 to another class of transmembrane molecules, Kremen (Krm) and promotes the internalization and inactivation of LRP6 (Mao et al., 2002) (Figure 5).

Reception of the Hh signal is initiated by binding to Patched (Ptc), a 12-pass transmembrane protein (Figure 5). A single *ptc* gene is found in *Drosophila*,

whereas there are to two different members in vertebrates, *ptc1* and *ptc2*. As a consequence of the Hh-Ptc interaction, Smoothened (Smo) is released from an inhibitory activity that is exerted by Ptc when it is not engaged by the ligand. Inhibition of Smo by Ptc may not depend on a direct binding, but rather by a catalytic activity of Ptc (Taipale et al., 2002). Moreover, Hh signal activates Ptc expression, establishing a negative feedback by sequestering Hh and reducing the range of signalling within a target field (Chen and Struhl, 1996).

In vertebrates, Hh signal also induces the expression of a cell-surface protein, Hedgehog Interacting Protein (HIP), which binds to and sequesters Hh (Chuang and McMahon, 1999) (Figure 5). Megalin, another member of the LRP family, has been shown to internalize Sonic Hedgehog (McCarthy et al. 2002) (Figure 5). Furthermore, *megalin* mutant mouse presents holoprosencephaly phenotypes resembling those of Shh mutant mice (Willnow et al., 1996).

Shifted (Shf), the Drosophila ortholog of the vertebrate Wnt inhibitory factor-1 (Wif-1), is a secreted molecule that interacts and co-localize with Hh in vivo (Figure 5). Mutations in *shifted* results in a reduced range of Hh signalling. Moreover, this activity requires the colesterol-modified form of Hh (Gorfinkiel et al., 2005). Previous studies indicated that vertebrate Wif-1 binds to Wnt proteins and blocks Wnt activity in *Xenopus* (Hsieh et al., 1999). Surprisingly, Shifted appears to have no effect on Wg range or activity, suggesting that Shifted is specific to Hh regulation.

The way in which Hh engages with its receptor has been subject to debate, but a consensus has emerged in which Hh binds to Patched (Ptc). However, the way Ptc regulates Smo activity remains a matter of conjecture. Moreover, Smo and Fz are closely related to each other, although the actual mechanisms of activation of Fz and Smo are fundamentally different. Remarkably, the presence of Wingless and Hedgehog on lipoprotein particles has intriguing implications for the function of LRPs. In fact, Kremen, Dickkopf and Wise, modulate Wnt signalling by interacting with LRPs.

Initiation of the intracellular transduction pathways

Once bound by their cognate ligands, the Fz/LRP and Ptc/Smo coreceptor complexes respectively activate the Wnt and Hh canonical signalling pathways. However, the mechanisms by which receptors couples to downstream effectors remains to be clarified. Some questions remaining are whether Wnt binding to Fz regulates a direct Fz-Dishevelled interaction or how Hh binding to Ptc leads to Smo activity de-repression.

The binding of Wnt to Fz/LRP leads to the phosphorylation of LRP by glycogen synthase kinase 3-β (Gsk3β) and casein kinase 1-γ (Cklγ), which is necessary for correct Wnt signalling (Zeng et al., 2005). Moreover, LRP5/6 interacts with the tumor suppressor protein Axin through five phosphorylated PPP(S/T) P repeats in the cytoplasmic tail (Mao et al., 2001). At the same time, Dishevelled (Dvl), an ubiquitously expressed cytoplasmic protein, interacts with a C-terminal intracellular motif in Fz (wong et al., 2003) and becomes phosphorylated upon Wnt signal initiation (Yanagawa et al., 2005) (Figure 6). Furthermore, Axin and Dvl contain a stretch of amino acids called the DIX domain that can homodimerize (Itoh et al., 2000). Phosphorylated Dvl, LRP6, Fz, Axin and GSK3 co-localized in LRP6 signalosomes and Dvl is required for LRP6 phosphorylation (Bilic et al., 2007). Altogether, this provides support for a model in which Wnt binding to Fz/lrp recruits a complex formed by Dvl–Axin–GSK3 to Fz. GSK3 and CK1γ can then phosphorylate the Axin docking residues of LRP, which allows binding of this scaffolding protein to LRP and inactivation of the protein complex that regulates β-catenin levels in the cell (Figure 6).

In the absence of the Hh ligand, Ptc inhibits Smo activity. Upon binding of the ligand, Ptc relieves its inhibition of Smo and allows Smo to activate downstream components. This relationship is evolutionarily conserved in both vertebrates and invertebrates. Biochemical data show that mammalian Ptch1 binds to the Shh ligand (Marigo et al., 1996; Stone et al., 1996) and genetic analyses show that Smo acts downstream of Ptc (Alcedo et al., 1996; Chen and Struhl, 1996; Zhang et al., 2001). It is, however, not clear how Ptc regulates Smo in any organism

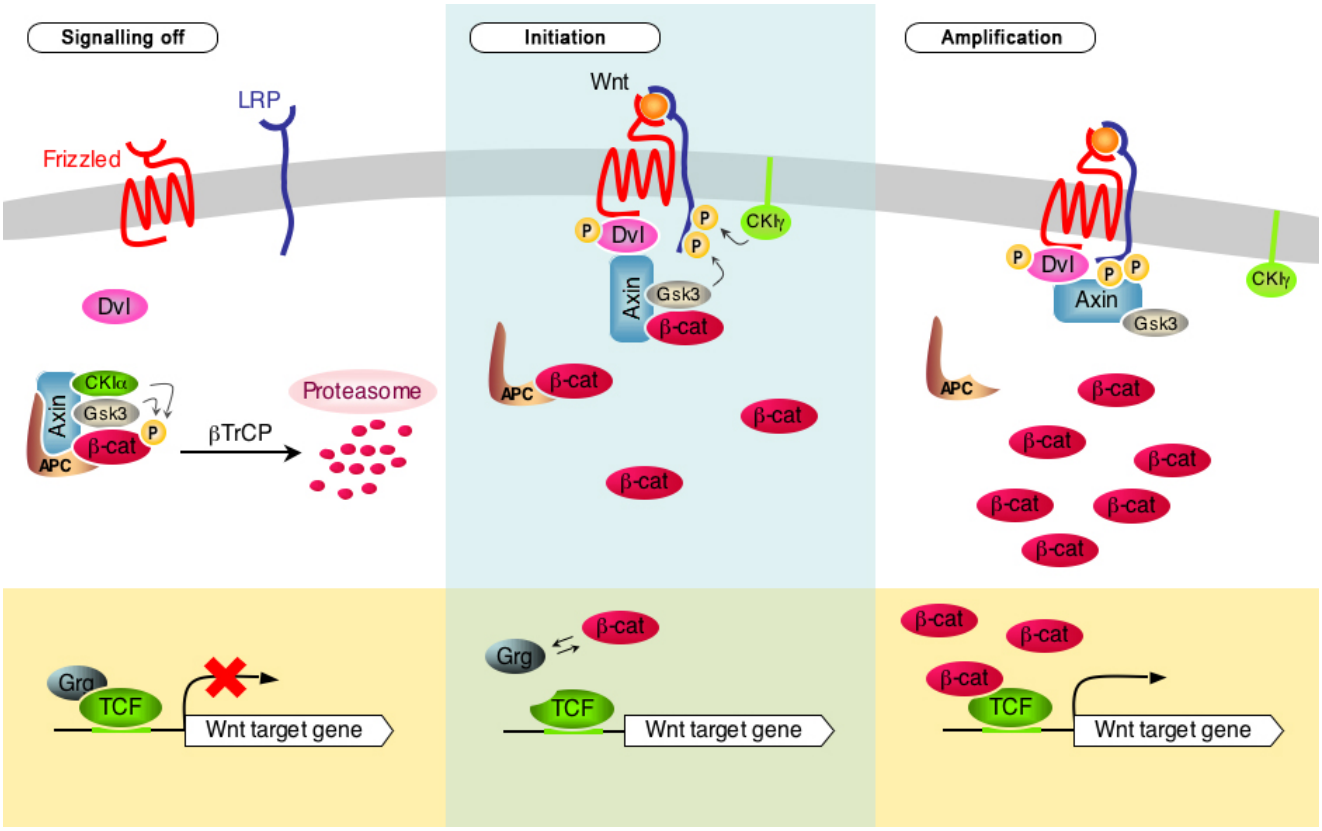

Figure 6. Model for the canonical Wnt transduction pathway. In the absence of a Wnt signal, β-catenin is phosphorylated and targeted for proteasome-mediated degradation by a destruction complex that contains Axin and Gsk3β among other proteins. On binding of Wnt to the receptors Frizzled (Fz) and LRP, dishevelled (Dvl) binds to Fz and recruits the destruction complex through interaction with Axin. Subsequently, Gsk3β phosphorylates critical sites on LRP, which, together with residues phosphorylated by CkIg, act as docking sites for Axin. Binding of axin to LRP leads to inhibition of the destruction complex and stabilization of β-catenin.

and there is little evidence that the endogenous Ptc and Smo proteins interact directly. In fact, activation of the Hh pathway induces opposite changes in the subcellular localization of Ptc and Smo (Denef et al., 2000; Incardona et al., 2002), and Ptc inhibits Smo activity in a catalytic manner, whereby one molecule of Ptc can regulate ~50 Smo molecules (Taipale et al., 2002) (Figure 7).

The *Drosophila* Smo protein has a large C-terminal tail that contains a set of sites for phosphorylation by protein kinase A (PkA) and casein kinase I (CkI). Smo phosphorylation is required for both its cell-surface accumulation and its activity in cell culture and in vivo (Jia et al., 2004; Zhang et al., 2004; Apionishev et al., 2005). However, the sequence of Smo cytoplasmic tail is highly divergent between vertebrates and flies, and most of the phosphorylated residues identified in the Drosophila are not conserved in vertebrates, which argue that vertebrate Smo proteins are differently regulated. Mammalian cell culture experiments show that Smo protein is also phosphorylated, and that the phosphorylation depends on a different kinase, the G-protein-coupled receptor kinase 2 (Grk2). Phosphorylation by Grk2 causes internalization of Smo, a process that also involves β-arrestin 2 (Arrb2) (Chen et al., 2004).

Cytoplasmic Complex

At the level of cytoplasmic transduction of the signal, Wnt and Hh pathways share distinct features. Wnt signals influence the cytoplasmic proteins that regulate β-catenin stability through several mechanisms, although it is unclear how this occurs. It is likely that Wnt-induced recruitment of Axin to the receptor complex, remove this scaffolding protein from the destruction complex, allowing β-catenin accumulation. On the other hand, increasing levels of Hh signalling promote binding of the scaffold protein Costal-2 (Cos2) to Smo cytoplasmic tail (Figure 7). This

interaction disrupts a cytoplasmic complex containing different kinases and releases the full length form of Gli transcription factors. These cytoplasmic complexes regulate phosphorylation, degradation and nuclear access of transcriptional regulators. In both cases, the kinases Ck1α and Gsk3β target the transcriptional regulator for ubiquitylation by Slmb; this is crucial for blocking signalling in the absence of ligand. Moreover, Suppressor of Fused (Sufu) is found in complexes with β-catenin as well as with Gli, and function as a negative regulator in both pathways, although the mechanisms by which SuFu functions remain unclear (Figure 7).

The central player in the canonical Wnt cascade is β-catenin, a cytoplasmic protein whose stability is regulated by the "destruction complex". In the absence of Wnt signalling, β-catenin is phosphorylated by the serine/threonine kinases, Casein kinase I (CkIα) and Gsk3 (Liu et al., 2002). The tumor suppressor protein Axin acts as the scaffold of this complex as it directly interacts with all other components: β-catenin, the tumor suppressor protein APC, and the two kinases, Ck1α and Gsk3β (Price, 2006). When Wnt receptor complexes are not engaged, Ck1α and Gsk3β sequentially phosphorylate β-catenin at a series of highly conserved Ser/Thr residues near its N-terminus. Phosphorylated β-catenin is then recognized by the F-box/WD repeat protein β-TrCP, a component of a dedicated E3 ubiquitin ligase complex. As a consequence, β-catenin is ubiquitinated and targeted for rapid destruction by the proteasome (Aberle et al., 1997). β-catenin mutant forms, that lack the phosphorylation sites required for its degradation, are Wnt unresponsive and can activate Wnt target genes constitutively leading to many different cancers (Giles et al., 2003).

It has also been suggested that protein phosphatases may regulate β-catenin stability as antagonists of the serine kinases. For example, heterotrimeric PP2A is required for the elevation of β-catenin levels that is dependent on Wnt (Yang et al., 2003). Moroever, PP2A can bind Axin and APC, suggesting that it might function to dephosphorylate CkI/Gsk3 substrates (Price, 2006). Finally, Dvl can interact with the destruction complex through the Gsk3β binding protein, GBP/Frat. Frat may promote the dissociation of Gsk3β from the degradation complex and prevent the phosphorylation of β-catenin (Li et al. 1999).

Genetic screens in *Drosophila* first identified Sufu as a suppressor of the Fused kinase, a positive regulator of Hh signalling (Pham et al., 1996). Later studies have shown that Sufu controls the nuclear-cytoplasmic distribution of Gli/Ci transcription factors through direct protein-protein interactions (Ding et al., 1999; Methot and Basler, 2000). Interestingly, Sufu is present in a complex with β-catenin and functions as a negative regulator of TCF-dependent transcription, most probably by regulating cytoplasmic-nuclear transport (Meng et al., 2001).

In the Hh pathway, Costal-2 (Cos-2) is a microtubule-binding and kinesin-related protein that physically interacts with *Drosophila* Smo (Jia et al., 2003; Lum et al., 2003; Ruel et al., 2003). A vertebrate ortholog of Cos2 has not been found yet. In the absence of Hh signalling, Cos2 protein is thought to act as a scaffold in a citoplasmic complex containing Fused (Fu), a serine–threonine protein kinase, PkA, Gsk3β, CkI and the Ci/Gli transcription factors. As with the degradation of β-catenin, this complex promotes the phosphorylation of Ci/Gli and, targets it for binding by Supernumerary limbs (Slimb), the Drosophila homolog of β-TrCP (Dai et al. 2003; Jia et al. 2005). This leads to ubiquitination by the SCF E3 ubiquitin ligase and partial proteolysis by the proteasome (Figure7). In response to low levels of Hh, the Smo-Cos2 complex is recruited to the membrane, and this relieves the inhibitory effect of Cos2 on Ci, which may lead to dissociation of Ci from the Smo-Cos2 complex (Ruel et al., 2003). However, Ci is not fully activated and cannot enter the nucleus, because Sufu tethers Ci in a complex that also includes Cos2 and retains it in the cytoplasm (Methot and Basler, 2000). At high levels of Hh signalling, this final restriction is removed, and Ci^{Act} can move into the nucleus to activate the pathway to a high level (Figure7).

Transcription factors

In the case of Wnt and Hh pathways the signal-dependent transcription factors function as repressors in the absence of signalling, but turn into activators of

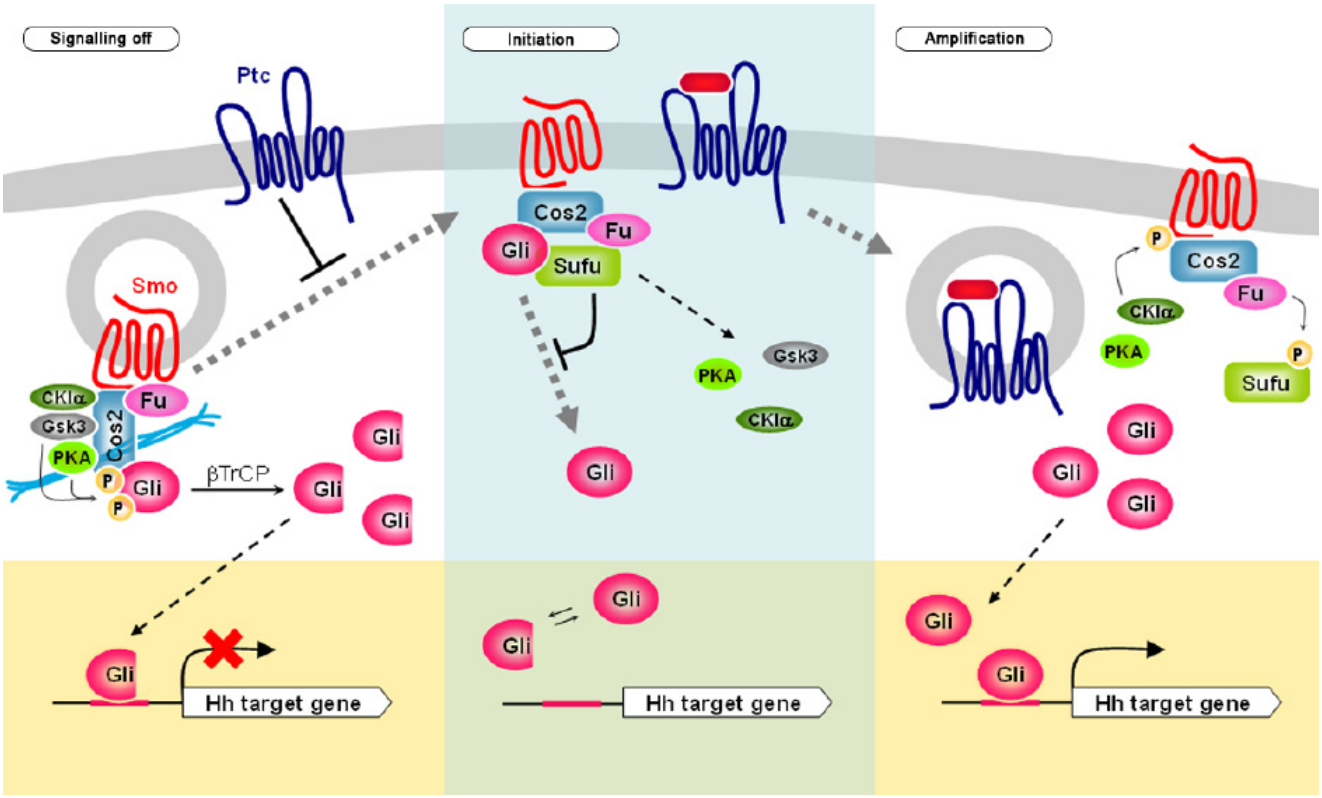

Figure 7. Schematic view of the Hh transduction pathway. In the absence of Hh, Ptc inhibits Smo and prevents its translocation to the cell surface. Cos2 binds microtubules and serves as scaffold for PKA, Gsk3 and CkI which phosphorylates Ci/Gli and target it for proteasomal degradation. Truncated Ci/Gli forms translocate to the nucleus and repress target gene expression.Initiation of signalling commence upon binding of Hh to Ptc that alleviates repression of Smo and allows its translocation from endosomes to the cell membrane. Cos2 complex with Smo release kinases and blocks Ci/Gli phosphorylation, although full-lenght Ci/Gli forms traslocation to the nucleus is slowed down by Sufu. Further activation of the pathway translocates Ptc from the cell membrale to lysosomes. Inhibition of Ptc results in phosphorylation of the cytosolic C-tail of Smo by PKA and CkI. Smo phosphorylation is crucial for full activation of the pathway and blocks Sufu activity, allowing translocation of full-lenght activator forms of Gli/Ci from cytoplasm to nucleus.

the same target genes upon ligand signalling.

The transcription factors are members of the TCF/LEF1 family in the Wnt pathway and of the Ci/Gli family in the Hh pathway. Degradation of β-catenin and of full-length Gli proteins is essential to maintain both pathways silenced by repressing transcriptional targets. Signalling activation disrupts β-catenin and Gli complexes sufficiently to prevent proteolysis but can also elicit further changes that increase the specific activities of both pathways.

Activation of Wnt signalling inhibits β-catenin phosphorylation and hence its degradation, allowing cytoplasmic accumulation. The elevation of β-catenin levels leads to its nuclear accumulation (Miller & Moon, 1997) and complex formation with T-cell specific factor/lymphoid enhancer binding factor-1 (TCF/LEF1) to transactivate transcription (Behrens et al., 1996; Molenaar et al., 1996). There is no consensus on the mechanism by which β-catenin translocates between the cytoplasm and the nucleus. However it is posible that β-catenin enters the nucleus without any partner since itself is closely related to importin/karyopherins and directly interacts with nuclear pore components (Stäedeli et al., 2006).

Whereas the fly and worm genomes both encode a single TCF protein, the vertebrate genome harbors four TCF/LEF genes (*TCF-1,-3,-4* and *LEF1*). These factors bind to the minor groove of the DNA helix, inducing a dramatic bend of over 90°. TCF target sites are highly conserved between the four vertebrate TCF/LEF proteins and *Drosophila* TCF. These sites resemble AGATCAAAGG (van de Wetering et al., 1997). Wnt/TCF reporter plasmids such as pTOP-flash (Korinek et al., 1997), widely used to measure Wnt pathway activation, consist of concatemers of 3–10 of these binding motifs cloned upstream of a minimal promoter. The different vertebrate TCF/LEF family members differ dramatically in their embryonic and

adult expression domains, yet they are highly similar biochemically, explaining the extensive redundancy unveiled in double knockout experiments (i.e. Galceran et al., 1999).

In the absence of Wnt signals, TCF acts as a transcriptional repressor by forming a complex with Groucho/Grg/TLE proteins (Cavallo et al., 1998; Roose et al., 1998). The interaction of β-catenin with the N-terminus of TCF (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997) transiently converts it into an activator, translating the Wnt signal into the transient transcription of TCF target genes. To accomplish this, β-catenin physically displaces Groucho from TCF/LEF (Daniels and Weis, 2005). The recruitment of β-catenin to TCF target genes affects local chromatin in several ways. Its C-terminus is a potent transcriptional activator in transient reporter gene assays (van de Wetering et al., 1997). It binds co-activators such as the c-AMP response element binding protein (CBP) and Brg-1, a component of the SWI/SNF chromatin-remodelling complex (Stäedeli et al., 2006).

On the other hand, zinc-finger proteins of the Ci/Gli family are the last effectors of Hh signalling pathway. The first *Gli* gene was identified as an amplified gene in a human glioma line (Kinzler et al, 1987). At present there are three well described *Gli* genes (*Gli1-3*) in vertebrates and a single gene, *cubitus interruptus (ci)* in insects. Gli proteins are large transcription factors of >1000 amino acids that bind DNA in a sequence-specific manner (Kinzler and Vogelstein, 1990), via the last three fingers of their five zinc-finger domain (Pavletich and Pabo, 1993). This zinc-finger domain is highly conserved but limited homology is present in other regions of the protein family. The most divergent member in the family is *Gli1* that lacks the N-terminal repressor domain and is thought not to be processed, keeping as a full-length form.

The processing of Ci/Gli gives rise to a truncated protein that contains the N-terminal repressor and zinc-finger domains, but lacks the C-terminal activator domain. A prerequisite for Ci proteolysis is the integrity of a cluster of phosphorylatable serines C-terminal to the zinc-finger domain (Price and Calderon, 2002). Corresponding sites are found in the Gli3 and are important to processing, indicating that this mechanism is likely to be conserved in vertebrates (Wang et al, 2000). Truncated forms are nuclear and have dominant-negative activity over that of full-length proteins. In the presence of Hh, the proteolytic processing of Ci/Gli in inhibited and the full-length protein enters the nucleus where it binds to the Gli-binding sites and activates the transcription of target genes. Gli1 itself is a direct target of the pathway (Dai et al, 1999) and is thought to transactivate through a VP16-like domain (Yoon et al, 1998).

Gli-mediated transcriptional control is regulated by the interaction of Ci/Gli proteins with distinct nuclear multiprotein complexes. Thus, Gli3 and possibly Gli2, like Ci, C-terminal domain interacts with the cAMP-response element binding protein (CBP) and may use it as a co-activator (Akimaru at al 1997; Dai et al, 1999). Otherwise, Gli3 repressor form directly binds to Ski and Sno corepressors, which act by recruiting a complex of histone deacetylases (HDAC). It has been reported that Ski is required for the transcriptional repression mediated by Gli3 (Dai et al, 2002).

The knowledge of the biochemistry and molecular biology of each pathway has grown in a way that allows in vivo approaches to cell responses to individual and combined signals. The Wnt and Hh families play multiple roles in CNS development. We use the early development of the CNS as a model to study signal integration, cell specification and proliferation.

Early Development of the CNS

Shortly after gastrulation, the ectoderm becomes partitioned into three different and separate cell populations that will have different fates. These are the epidermal ectoderm, which will become primarily epidermis, the neural crest, which cells migrate to form a portion of the peripheral nervous system as well as a variety of non-neural cell types, and the neural ectoderm, which comprises the central nervous system (CNS). Many tissues interact during neurulation to induce and regionalize the neural plate and to produce the morphogenetic forces that drive neurulation (Smith and Schoenwolf, 1997).

The early neural plate is initially rostral in character

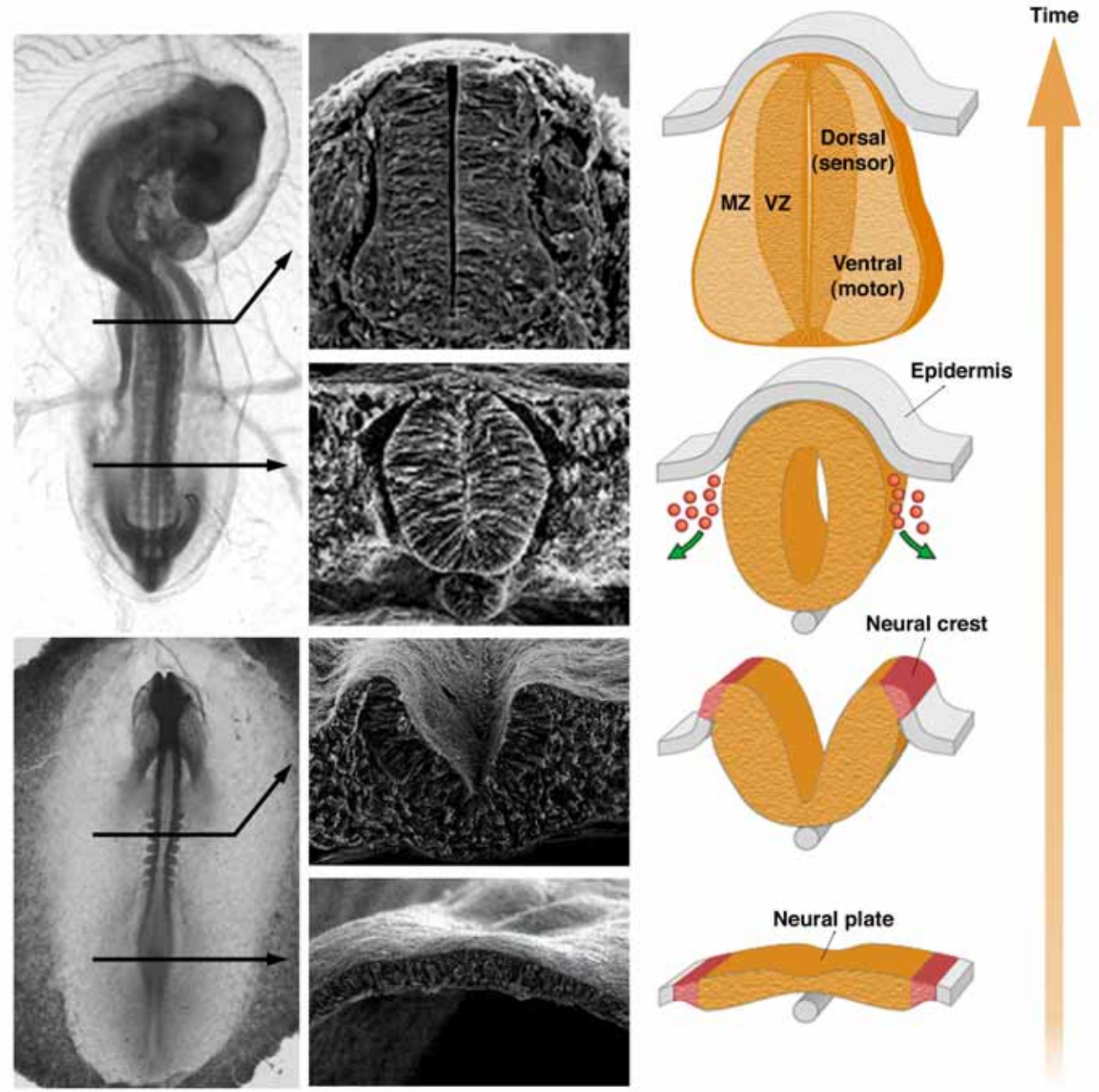

Figure 8. Representation of morphological changes and anayomical/functional regionalization during neural tube formation in the chicken embryo.

and more caudal regions, including the spinal cord, form as a result of the caudal regression of the organizer (Hensen's node in the chick). This anterior-posterior regionalization is in part controlled by Wnt signalling (i.e. Nordstrom et al., 2002) and ends up with two structural and functionally different structures: a rostral or cephalic region, where the brain will form; and a caudal region that give rise to the spinal cord. Together with AP cell specification processes, changes in shape of the neural tube take place. In the cephalic region, the wall of the tube grows to create a series of swellings and constrictions that define the various brain compartments, while the neighbor caudal region, the developing spinal cord, remains as a simple tube that elongates concomitantly to caudal body axis extension.

The spinal cord is the anatomically simplest and most conserved region of the vertebrate CNS and it is an ideal model for studying the developmental regulation of growth and pattern formation. When the

neural tube first forms, its walls are composed of bipolar shaped cells spanning the entire width of the tube that conform a typical pseudostratified epithelium. At this early stage, majority of the cells proliferate defining the germinal neuroepithelial layer known as ventricular zone (VZ) (later ependyma). Shortly after neural tube closure, scattered neural precursors stop dividing and detach from the apical luminal side of the tube. As a consequence, these postmitotic cells commence differentiation and move basally through the neural epithelium. The region of the neural tube, peripherally to the replicating neuroepithelial cells, where these cells accumulate and terminally differentiate into both neurons and glia (astrocytes and oligodendrocytes) is called the mantle zone (MZ) (Boulder Committee et al., 1970) (Figure 8).

There is good evidence that cell replication and withdrawal from the cell cycle occur at defined times during development. Typically in vertebrates, neurons in the hindbrain are the first to exit cell cycle, followed in close succession by the first neurons in the spinal cord and in the ventral mesencephalon. Moreover, as the CNS matures a further anatomical and functional subdivision appears along the DV axis. In general, the first-born neurons contribute to ventral CNS structures (e.g. motor nuclei), whereas dorsal structures that perform sensory and integrative functions are populated by neurons produced with a delayed time in development (Hollyday, 2001).

Dorsoventral patterning of the spinal cord

As a result of studies on amphibians from the 1920s onwards, it became clear that the surrounding/underlying mesoderm was the major determinant of the dorsoventral organization of the neural tube, rather than it being due to any intrinsic self-organizing capacity. For example, in the absence of the notochord, the characteristically thin floor plate is missing and a thick mass forms on its place (reviwed by Holtfreter and Hamburger, 1955). The same principles were reinforced in subsequent experiments on chick embryos from the 1950s, and in recent years considerable progress has been made in determining the cellular and molecular events that control dorsoventral patterning of the neural tube (Briscoe and Ericson, 2001).

During rostrocaudal extension of the neural plate and elaboration of the pattern in the AP axis, DV patterning is simultaneously taking place. Although roof plate and floor plate cells do not directly participate in neurogenesis, these groups of cells form important embryonic organizing centers that provide the inductive signals necessary to specify the spatial coordinates of a precursor cell along the DV axis. Selective removal of the roof plate results in the loss of the most dorsal interneurons (Lee et al., 2000), while taking away the notochord results in the loss of ventral phenotypes (van Straaten and Hekking, 1991).

In the dorsal neural tube, a gradient of Bone Morphogenetic Proteins has been proposed to extend throughout the DV axis of the neural tube providing the positional information necessary for the generation of the different dorsal and intermediate neuronal cell types (Nguyen et al., 2000). Moreover, BMP4, BMP7, Dorsalin and Activin are involved in the specification of the dorsal populations of the neural tube (Liem et al., 1997). However, the redundancy among the distinct TGF-β family members expressed in the dorsal neural tube, together with the possible interactions with other pathways, such as the canonical Wnt pathway (Chesnutt et al., 2004), keep the mechanism that drive dorsal patterning far from be resolved.

In the ventral neural tube, it has been demonstrated that Shh mediates the specification of the floor plate induced by the notochord (Chiang et al., 1996; Echelard et al., 1993; Roelink et al., 1994). Once specified, the floor plate cells secrete Shh that is both sufficient and necessary for the emergence of the different ventral neuronal subtypes (Echelard et al., 1993; Martí et al., 1995). In neural tube explants, different concentrations of recombinant Shh protein give rise to distinct ventral neural fates in a way that correlates with the distance from the Shh source that these neuronal subtypes set out in vivo (Ericson et al., 1997) (Figure 9).

Together these inductive signals regulate the expression patterns of different transcription factors (Pax6, Pax7, Dbx1, Irx3, Nkx2.2 or Olig2, among others) in partially overlapping domains within the ventricular epithelium (Briscoe and Ericson, 2001). The expression profile along the DV axis of these

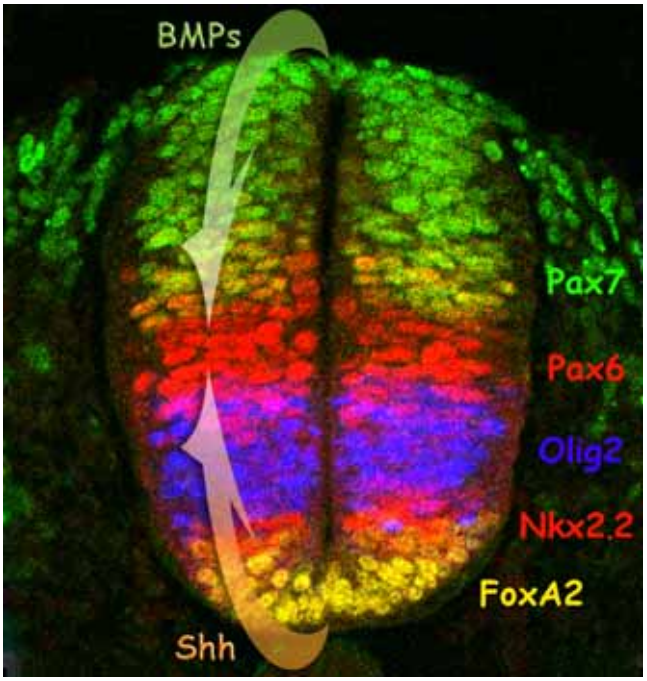

Figure 9. Expression of dorsoventral patterning genes in the ventricular zone. The floor plate secretes Shh and the roof plate secretes BMP signals. These secreted factors act in opposing gradients to pattern the spinal cord by acting on patterning genes in different DV territories. The patterning genes Pax7, Pax6, Olig2, Nkx2.2 and the floor plate marker FoxA2, are expressed in nested dorsal to ventral domains.

transcriptional regulators control the genetic network necessary for the proper anatomical segregation of each neuronal subtype (Lee and Pfaff, 2001). Thus, cutaneous sensory neurons form circuits in the dorsal spinal cord while visceral and somatic motor neurons are found largely in the ventral spinal cord (Jessell, 2000). Connecting these two are several interneuron populations that form distinct axonal trajectories and circuits. There are now many molecular markers of neuronal populations in the spinal cord that have been extensively used for studies of DV patterning and have resulted in the identification of further sub-groupings. The dorsal interneurons are now subdivided into 6 groups, while ventral neurons are divided into 4 groups of interneurons and motorneurons. Distinct gene expression profile identified each domain.

Sonic hedgehog controls the ventral pattern formation of the CNS

The gradient of Shh secreted from the notochord and floor plate controls the specification of five molecularly distinct classes of ventral progenitors. Each neuronal subtype is generated from a spatially discrete progenitor domain (p3, pMN, p2, p1, p0), and these distinct domains are defined by the expression of a characteristic combination of genes that encode Homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors.

These markers can be subdivided into two classes on the basis of their pattern of expression and mode of regulation by Shh. Class I proteins (Pax7, Pax6, Dbx1, Dbx2, and Irx3) are constitutively expressed by neural progenitor cells and their expression is repressed by distinct threshold concentrations of Shh. As a consequence their ventral boundaries delineate progenitor domains. Conversely, the expression of each Class II protein (Nkx2.2, Nkx6.1, Nkx6.2, and Olig2) requires Shh signalling and is achieved at distinct Shh threshold concentration. Thus their dorsal boundaries delineate progenitor domains (Briscoe and Ericsson, 2001).

However, the different transcriptional responses to graded Shh signalling, cannot adequately explain the appearance of sharp boundaries of Class I and Class II gene expression patterns observed in vivo (Briscoe et al., 2000). Gain and loss of function experiments suggest that selective cross-repressive interactions between distinct pairs of Class I and Class II proteins that abut the same progenitor domain, function to refine such boundaries and serve to consolidate progenitor domain identity (Lee and Pfaff, 2001) (Figure 10).

Furthermore, the three members of the Gli family in vertebrates are expressed in different regions of the neural tube and it has been shown that different levels of Gli activity in vivo are sufficient to mimic the full range of responses to Shh in the neural tube (Stamataki et al., 2005). This data suggest that the graded Shh signal is transduced into a gradient of Gli activity that drives patterning of the ventral neural tube.

Wnt/β-catenin signalling regulates dorsal specification of the CNS

The only evidence for a role of Wnt signalling in the control of D/V patterning of the spinal cord comes from the analysis of the Wnt1$^{-/-}$; Wnt3a$^{-/-}$ compound

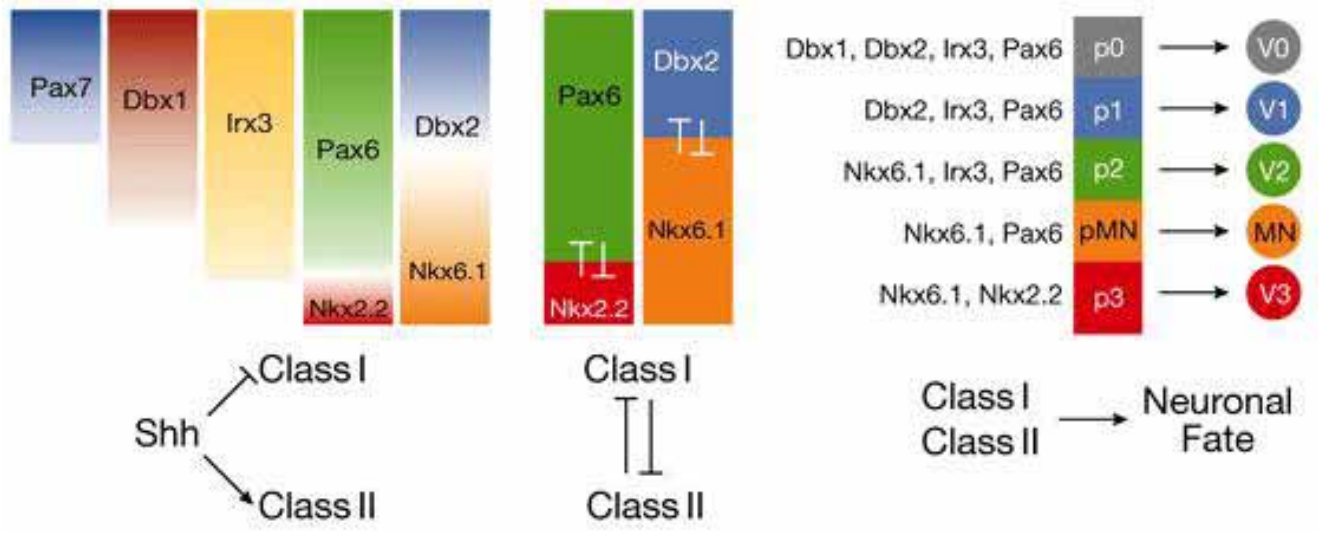

Figure 10. Ventral neural progenitor domains of the spinal cord are defined by Sonic hedgehog-regulated combinatorial expression of transcription factors. The concentration gradient of Shh regulates the ventral expression domains of a series of transcription factors in ventral progenitor (p) cells. Three aspects are crucial to this system: Shh either represses (class I genes) or induces (class II genes) expression at different concentration thresholds (left). Progenitor gene-expression domains are refined and maintained by negative cross-regulatory interactions between those proteins that share a boundary (centre). The combinatorial expression of homeodomain proteins in distinct progenitor domains determines the neuronal subtype that arises from each domain (right) (Jacob and Briscoe, 2003).

mutant mice, where the three dorsal most subtypes of interneurons, dI1, dI2 and dI3, are reduced, while the number of cells in dI4 to dI6 interneuron populations is doubled and its domains are expanded dorsally into what would normally be the dI1 to dI3 domains. Additionally, Wnt3a induces dI1 to dI3 interneurons and represses dI4 to dI6 interneuron differentiation in vitro (Muroyama et al., 2002). However, $Wnt1^{-/-}$; $Wnt3a^{-/-}$ double mutant mice do not necessarily tell the complete story of the role for Wnts in DV patterning. The reduction of dI1 to dI3 interneurons occurred in $Wnt1^{-/-}$; $Wnt3a^{-/-}$ double mutant embryos but not in $Wnt1^{-/-}$ or $Wnt3a^{-/-}$ single mutants, indicating that the expression of each of these Wnt proteins alone is sufficient to generate normal populations of dI1, dI2 and dI3 interneurons. Moreover, the inactivation of Wnt3, the most widely expressed of the dorsal neural Wnts (Roelink and Nusse, 1991; Parr et al., 1993), results in gastrulation defects and death before DV pattern formation can be evaluated (Liu et al., 1999). Therefore, Wnt1, Wnt3a, and Wnt3 may be acting redundantly in the control of dorso-ventral patterning of the neural tube. Additionally, Wnt/β-catenin activity appears also to control expression of dorsal markers in the mouse telencephalon, extending this activity to the anterior CNS (Backman et al., 2005) (Figure 11).

Thus, although knowledge in DV pattern has advanced in last few years in the analysis of simple response, there is still much to learn about signal integration and how a particular progenitor cell reads and interprets multiple morphogenetic signals for cell fate decisions.

Growth in the neural tube

In parallel to the specification of the different subtypes of neural precursors, the growth of the neural tube in the three axis of the body is taking place. A tight control of this process is required in order to generate the correct number of neural and glial cells. The size of any organ is determined by the number of its constituent cells and the average size of each cell. Both of these features are controlled and linked by developmental pathways to components of the cell cycle.

The nucleus of mitotically active neural precursors moves within the cytoplasm, back and forth across the wall of the tube in a process called interkinetic nuclear migration. A key feature of this process is that nuclear position varies in relationship to the phases of cell cycle. While mitotic figures are found only adjacent to the lumen of the neural tube, cells in S-phase have nuclei located in the outer half of the neural epithelium.

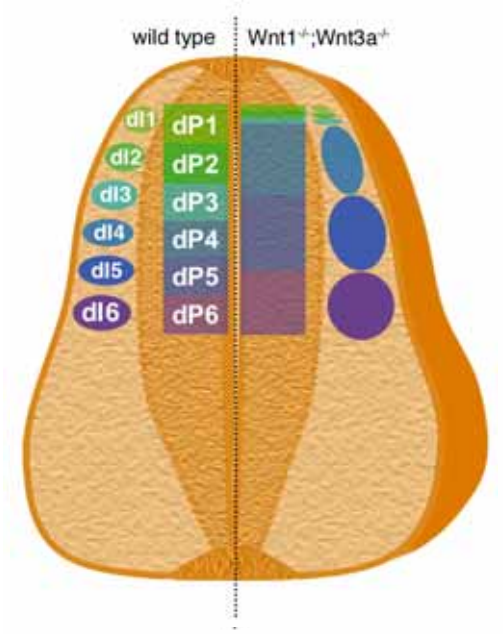

Figure 11. Summary of the phenotype of dorsal interneuron development in Wnt1$^{-/-}$;Wnt3a$^{-/-}$ embryos. Absence of Wnt1 and Wnt3a (right) led to diminished development of dorsalmost subtypes of interneurons, dI1, dI2 and dI3 and to a compensatory increase of dI4 to dI6 interneurons populations.

This process was first deduced by Sauer (Sauer, 1935) from measurements of the nuclear volume, and was later directly demonstrated by using incorporation of thymidine analogous into DNA (Fujita, 1964) (Figure 12).

The overall size of the CNS is governed by cell cycle machinery. This is clearly demonstrated by the enlarged brains and retinas of mice lacking the rather ubiquitous cell cycle inhibitor p27Kip1 (Nakayama et al., 1996). Progression through the cell cycle depends on the sequential formation, activation, and subsequent inactivation of a series of cyclins and cyclin-dependent kinase (CDK) complexes (Sherr and Roberts, 1999; Zhang, 1999). The transition from G1 to S involves the activation of several E2F transcription factors that induce the expression of genes necessary for the initiation of S-phase, including E and A-type cyclins. During the first part of G1, E2F transcription factors are kept in a transcriptionally inactive state because they are associated with the retinoblastoma tumor suppressor gene product (pRb). Phosphorylation of pRb protein by cyclin-dependent kinases releases E2F from inhibition and transcription of E2F-dependent genes triggers progression into and through S-phase. Cyclin-dependent kinases cdk2, cdk4 and cdk6 are expressed during G1. They interact with D-type, E-type and A-type cyclins to hyperphosphorylate pRb and release free E2F. The transition from G2 to M is controlled by the cyclin-dependent kinase cdk1 (also called cdc2), and its regulatory subunits, A-type and B-type cyclins (Figure 12).

Control of G1-S transition is mediated by D-type Cyclins. D-type cyclins complexed with Cdk4 or Cdk6 carry out the initial phosphorylation of pRb that leads to the de-repression of cyclins A and E. The activity of cyclinE and A-Cdk2 drives the cell into S phase. The major function of the D-type cyclins is to provide a link between mitogenic cues and the potentially autonomous cell cycle machinery.

Superimposed upon this core circuit are the Cdk inhibitors (CKIs) that are thought to be induced by negative or anti-mitotic growth signals. Induction of CKIs will prevent phosphorylation of pRb by Cdks, and keep the cells in G1. Cells kept permanently in G1 are said to be in G0.

Expression of genes whose proteins control progression of the cell cycle has been demonstrated in various regions of the developing neural tube, while the signals that induce either the cyclins or CKIs are just starting to be identified. A number of proteins with mitogenic actions on the ventricular neural epithelium have been identified including Wnt1 (Dickinson et al., 1994), Fibroblast Growth Factor-2 (Dono et al., 1998; Ortega et al., 1998) and Sonic hedgehog (Rowitch et al., 1999). However, the molecular mechanisms that permit to connect these extracellular signals to the cell cycle engine remain poorly understood.

The Wnt canonical pathway maintains precursor cell proliferation at the ventricular zone

Several lines of evidence have implicated Wnt proteins in the regulation of growth in multiple areas of the developing CNS. The midbrain and the hippocampus are absent in Wnt1$^{-/-}$ mice (McMahon and Bradley, 1990; Thomas and Cappechi, 1990) and Wnt3a$^{-/-}$ mice (Lee et al., 2000), respectively, while Wnt1$^{-/-}$; Wnt3a$^{-/-}$ double mutant mice have an additional reduction in caudal diencephalon, rostral hindbrain and spinal cord (Ikeya et al., 1997; Muroyama et al., 2002), suggesting that

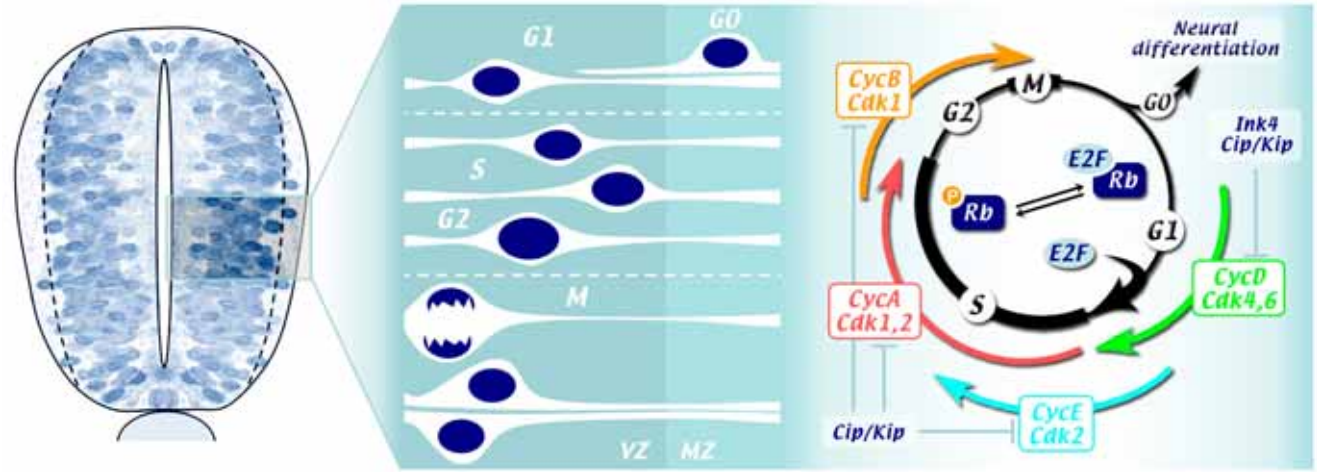

Figure 12. Summary diagram illustrating cell cycle dynamics in neural progenitors. The nucleus of proliferating cells undergo interkinetic nuclear migration that is coordinated with phase of the cell cycle. Nucleus move away from the ventricular surface during G1 and undergo DNA synthesis (S) in the outer half of the ventricular zone (VZ). They return to the ventricular surface during G2, where they undergo mitosis (M), after completing mitosis and re-entering G1, cells can exit cell cycle and differentiate, or they can re-enter the cell cycle. The basic mechanism of the cell cycle is shown with several of the key checkpoints and regulators.

both genes play broad semi-redundant roles in growth control of the CNS. Consistent with a prominent role in precursor cell proliferation, ectopic expression of Wnt1 (Dickinson et al., 1994; Megason and McMahon, 2002; Panhuysen et al., 2004) and Wnt3a (Megason and McMahon, 2002) causes overgrowth without apparent alterations in cell identities along the dorso-ventral axis of the neural tube. Other Wnt genes are expressed at different dorso-ventral levels in the developing neural tube (Parr et al., 1993; Hollyday et al., 1995) but there is no data at the moment supporting a role in the control of the neuroepithelial growth.

Evidence that the Wnt-dependent control of neuroepithelial cell proliferation is mediated through the canonical Wnt-pathway 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). Ablation of β-catenin show that the tissue mass of the spinal cord and several brain areas, including cerebral cortex and hippocampus, is reduced and that the neural precursor population is not maintained. In contrast, the spinal cord of chick and the brain of mice that express activated β-catenin have an enlarged mass with an increased population of neuronal precursors (Megason and McMahon, 2002; Zechner et al., 2003). β-catenin signals thus seem essential for the maintenance of proliferation of neuronal progenitors, controlling the size of the progenitor pool, and impinging on the decision of neuronal progenitors to proliferate or differentiate.

Direct targets of the Wnt canonical pathway are defined as those with functional TCF binding sites. Two key regulators of the G1/S transition of cell cycle meet these requisites: cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999) and c-Myc (He et al., 1998). Transcriptional activation of cyclin D1 through the β-catenin/TCF pathway also occurs in the developing neural tube, suggesting that Wnt signalling positively regulates cell cycle progression and negatively regulates cell cycle exit of spinal cord precursors, in part through transcriptional regulation of cyclin D1 (Megason and McMahon, 2002; Panhuysen et al., 2004).

Is therefore the Wnt/β-catenin-mediated regulation of cell cycle progression sufficient for the proper growth of all the different CNS areas and the generation of the proper number of precursor cell populations? β-catenin is ubiquitously expressed in the developing spinal cord but mitogenic Wnts are only expressed in the most dorsal region of the neural tube. The unanswered question is then whether this mitogenic dose is sufficient to maintain proliferation of precursor cells all along the dorsoventral axis by transcriptional activation of cyclin D1.

The Hedgehog pathway controls growth of the CNS

In addition to this fundamental role played in pattern formation of the ventral CNS, the Shh-Gli pathway has been demonstrated to play a major mitogenic role in the development of rostral brain structures, including the cerebellum (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999), neocortex and tectum (Dahmane et al., 2001; Palma and Ruiz i Altaba, 2004). Shh-mediated proliferation is also required for the maintenance of neural stem cells in late development and adult CNS (Lai et al., 2003; Machold et al., 2003).

The lack of Shh activity either by direct notochord removal or by genetic inactivation of Shh or other components of the pathway results in a decrease size of the spinal cord (Straaten and Hekking, 1991; Chiang et al., 1996). On the other hand, activation of Shh pathway increases progenitor proliferation and consequently the growth of the neural tube both in mouse and chicken. For instance, neural tubes of mice lacking the ligand binding inhibitor HIP-1 are enlarged (Jeong and McMahon, 2005). Moreover, the mitogenic role of Shh in the neural tube has been recently shown to be mediated by a Gli transcriptional activity (Cayuso et al., 2006).

Although these experimental data strongly suggest a mitogenic role for Shh in the early developing neural tube, the molecular interpretation of Shh-mitogenic activity still poorly understood. Transcriptional activation of regulators of G1/S transition cyclinD1 (Ishibashi and McMahon, 2002, Lobjois et al., 2004) and N-Myc (Kenney et al., 2003) seems to be as well dependent on Shh activity, suggesting a conservation of the molecular machinery regulating cell cycle progression of early neural precursors. Moreover, Shh signalling upregulates G2/M transition component cdc25b in the neural tube (Bénazéraf et al., 2006).

The chicken model system

To look for the integration of Wnt and Shh signalling pathways during the morphogenesis of the neural tube we have selected the chicken embryo as the principal experimental model. The chick embryo has

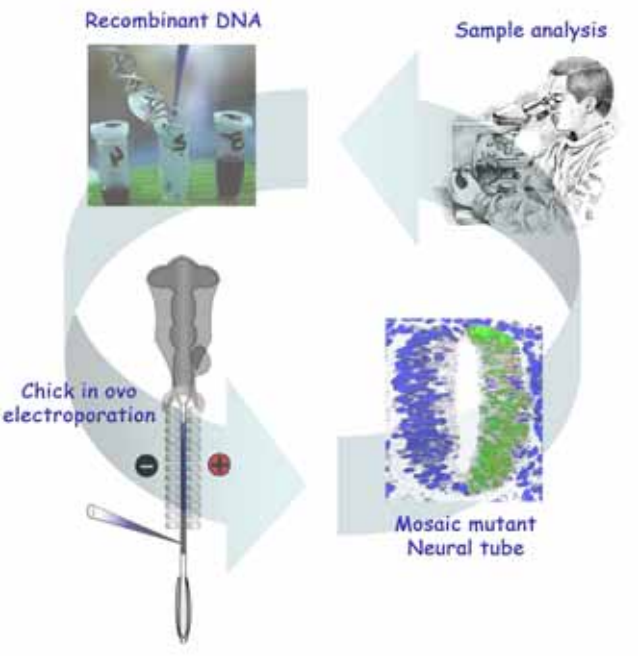

Figure 13. Schematic representation of chicken in ovo electroporation.

a long history as a mayor model system in developing biology and it is a simple and amenable in vivo model that can be genetically manipulated in an easy way by in ovo electroporation (Krull, 2004; Stern, 2005). The combined use of overexpression plasmids, as DNA vehicles, and in ovo electroporation give rise to a transient and mosaic transfected embryo. This allows performing gain or loss of function in vivo assays with single cell resolution in a time and space controlled way (Figure 13).

During neural tube development, neural progenitors proliferate to generate sufficient neural precursors for the construction of a functional nervous system. At the same time, these progenitor cells are taking important cell fate decisions so that each progenitor pool contains sufficient of each type. All these processes can be easily identified by the use of specific molecular markers, therefore making the chicken an ideal and efficient model of study.

OBJECTIVES

The research in this doctorate thesis was aimed at understanding several aspects of early development of the spinal cord including the regulatory networks that control the balance between dorsal and ventral character of the neural tissue and the networks that control proliferation of neural progenitors.
In particular I have focused in two main objectives:

1.- To study the role of the Wnt canonical pathway in dorsalization of the spinal cord, and its possible interaction with the ventralizing activity of Sonic hedgehog.

1.a.-Expression and functional analysis of the TCF transcription factors
1.b.-Interaction of the Wnt pathway with other signals known to control DV patterning
1.c.-Search for possible Wnt-target genes regulating DV patterning

2.- To study the role of the Wnt canonical pathway in the growth control of the neural tissue and its possible interaction with the proliferative activity of Sonic hedgehog.

2.a.-Analysis of the Wnt activity in control of proliferation
2.b.-Interaction of the Wnt pathway with Shh activity in the regulation of G1/S transition
2.c.- Analysis of the Wnt and/or Shh activities in the regulation of the G2/M transition

La vía canónica de Wnt restringe el gradiente de actividad Shh/Gli a través del control de la expresión de Gli3 para establecer el patrón dorsoventral del tubo neural

La formación del patrón dorsoventral dorsoventral del sistema nervioso central en vertebrados está controlada por la acción de señales morfogenéticas. Estas señales son secretadas por centros de señalización situados en el extremo dorsal, ectodermo y placa del techo, y ventral, notocorda y placa del suelo, del tubo neural. La vía de señalización de Shh/Gli juega un papel principal in el establecimiento temprano de la región ventral del tubo neural. Sin embargo, los mecanismos moleculares que restringen la expresión de los genes responsables de establecer este patrón a un dominio concreto no son del todo conocidos. En este trabajo mostramos que las señales morphogenéticas Wnt1 y Wnt3a, activando la vía de señalización canónica mediada por β-catenina/Tcf, regulan la expresión de genes dorsales y reprimen el programa ventral, mediante un mecanismo que depende de la actividad Gli. Además, mostramos que la expresión de Gli3 está controlada por la vía de Wnt. La identificación y caracterización de regiones no codificantes altamente conservadas alrededor del locus de *Gli3* humano revela que contienen sitios de unión consenso para los factores Tcf/Lef-1 activos. Esto indica que la expresión dorsal de Gli3 está controlada directamente por la actividad de la vía canónica de Wnt. A su vez, Gli3, actuando como un represor transcripcional, restringe la actividad del gradiente ventral Shh/Gli para establecer el patrón dorsovantral del tubo neural correctamente.

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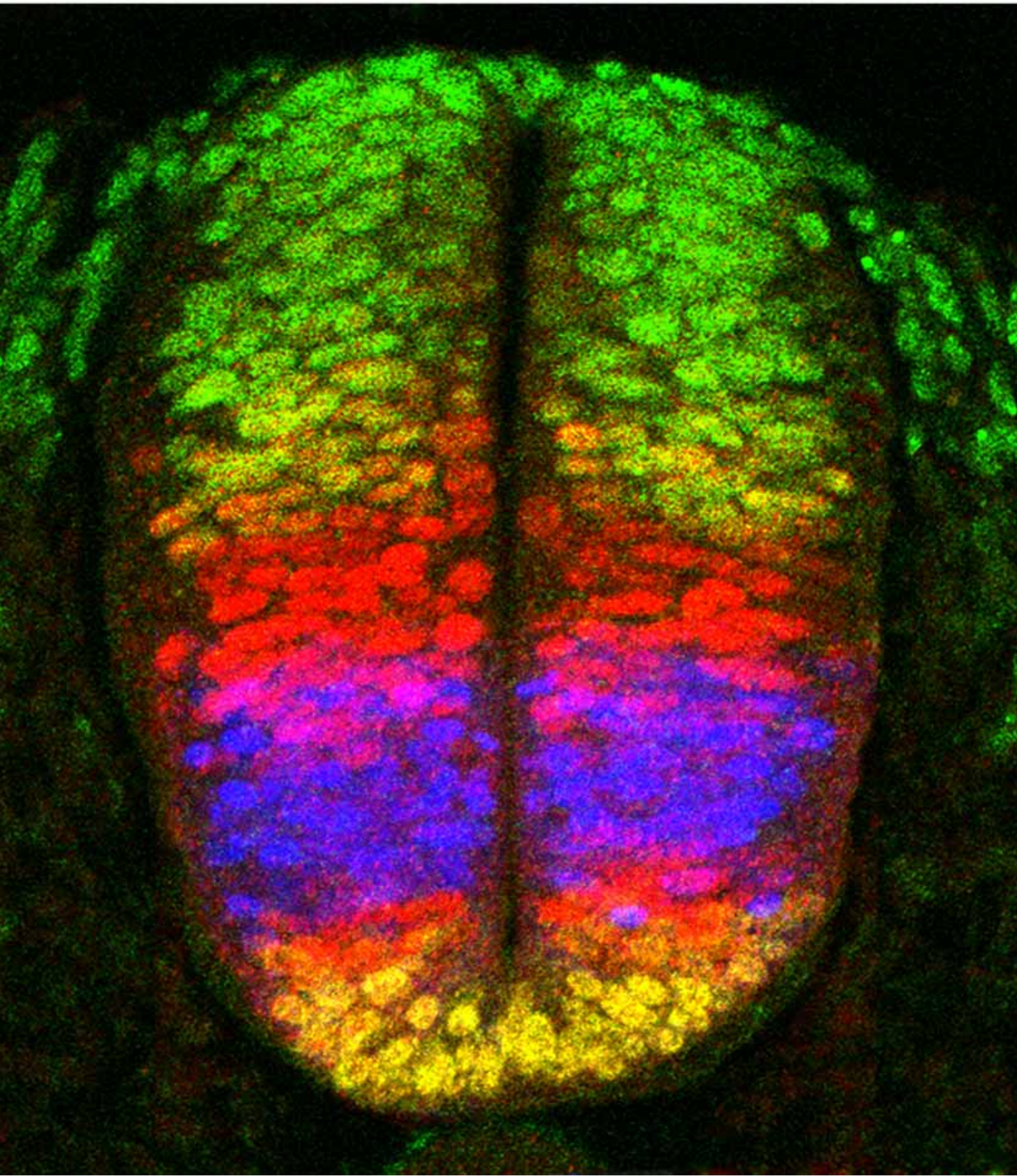
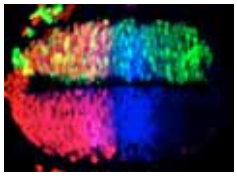


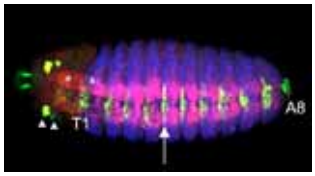
Outside the gonad: a story of p53 and cell death

In animal embryos, primordial germ cells (PGCs) migrate long distances to the developing gonads. During this journey, programmed cell death removes abnormal, misplaced or excess cells. The regulation of this process, which ensures germline integrity, is poorly understood. Now, Clark Coffman and co-workers report that the tumour suppressor p53 and Outsiders (a monocarboxylate transporter) regulate programmed cell death during PGC development in *Drosophila* (see p. 207). They show that in loss-of-function *p53* and *outsiders* embryos, the programmed cell death (but not migration) of PGCs is abnormal, resulting in the persistence of ectopic PGCs outside of the gonads. This *p53* phenotype (the first developmental phenotype seen for loss of *Drosophila p53* function) closely resembles that of *outsiders* mutants, note the researchers. Furthermore, overexpression of *p53* in the PGCs of *outsiders* embryos partly suppresses the cell death phenotype. Thus, p53 and Outsiders may function in a common pathway to eliminate a subset of PGCs during embryogenesis.



Wnt-Shh neural tube patterning crosstalk

In the developing spinal cord, morphogenetic signals secreted from dorsal and ventral signalling centres control dorsoventral (DV) patterning. The Shh/Gli pathway plays a major role in patterning the ventral neural tube but what restricts its activity to specific domains? On p. 237, Alvarez-Medina and colleagues propose that the Wnt canonical pathway fulfils this role. Wnt1 and Wnt3a, which signal through the canonical β-catenin pathway, are expressed in the dorsal midline region of chick embryos. Their misexpression along the DV axis by in ovo electroporation, the authors report, expands dorsal marker gene expression in the developing neural tube, whereas their inhibition suppresses the dorsal programme and expands ventral gene expression. These phenotypes, the authors show, depend on the Wnt-controlled expression of Gli3, which in its repressor form (Gli3R) acts as the main transcriptional repressor of the Shh/Gli pathway. Together, these observations suggest that the Wnt canonical pathway indirectly restricts graded Shh/Gli ventral patterning activity by regulating the dorsal expression of Gli3 to ensure proper spinal cord patterning.

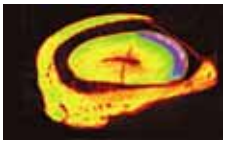


Heads down with Hox

Conserved Hox transcription factors direct the formation of distinct structures along the anteroposterior axis of bilaterian animals. Given that Hox genes probably all derive from a single unique gene by duplication, might they also share a common function? On p. 291, Coiffier and co-workers propose that this is the case by showing that all *Drosophila* central and posterior (CP) Hox genes repress head formation in the fly's trunk, in addition to their well-known roles in segment identity. Hox genes of many species fall into CP and anterior classes based on their expression pattern and sequence similarities. The researchers report that, in *Drosophila*, the central Hox proteins (including Antennapedia and Ultrabithorax) and the posterior Hox protein Abdominal B prevent the expression of the head-specific gene *optix* in the trunk. Furthermore, several non-Hox genes, including *Teashirt* and *Wingless/Wnt*, contribute to this repression. The researchers propose, therefore, that an early function of Hox genes was to repress the head and that novel Hox functions that specialise the trunk appeared later.

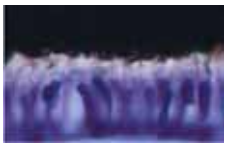
Network theory unravels patterning

Pattern formation during development depends on combinatorial interactions between signalling pathways, but the strategies for pathway integration and coordination are still poorly understood. Now Yakoby and colleagues have developed a new model based on network theory to explain how the *Drosophila* eggshell is patterned (see p. 343). During *Drosophila* oogenesis, the EGFR and Dpp pathways specify the follicle cells that give rise to dorsal eggshell structures. Follicle cells that express the transcription factor Broad (Br), whose expression is regulated by both EGFR and Dpp signalling, form the roof of these structures. From their observations of signalling patterns during eggshell formation and from published data, the researchers propose that EGFR signalling determines the spatial pattern of Br by inducing the expression of both *br* and its transcriptional repressor Pointed (a feedforward loop). Later, a feedback loop activated by Br controls Dpp, which terminates Br expression. Future work will explore how other feedback loops interact with the simple regulatory network motifs described in this new model to generate complex gene expression patterns.



Sprouty free and long in tooth

Unusually for mammals, rodent incisors grow continuously, fuelled by stem cells in their mesenchymal and epithelial compartments. Constant abrasion of the incisor's lingual side (the side facing the tongue), which unlike the opposite side has no hard enamel covering, maintains its length and shape. But why is enamel produced asymmetrically? On p. 377, Gail Martin and co-workers report that sprouty (Spry) genes, which encode FGF signalling antagonists, ensure this asymmetrical enamel deposition and prevent the growth of tusk-like incisors. The researchers show that enamel-producing ameloblasts develop from stem cells on both sides of the incisors of *Spry4*$^{-/-}$ mouse embryos and that an ectopic epithelial-mesenchymal FGF signalling loop on the lingual side of the incisors causes this phenotype. Interestingly, ectopic ameloblast formation is maintained after birth only if the dosage of *Spry1* or *Spry2* is also reduced. Thus, the researchers suggest, the generation of differentiated progeny (such as ameloblasts) from stem cell populations can be differentially regulated in embryos and adults.



Vesicle trafficking: transported into development and disease

Several inherited human diseases [such as arthrogryposis-renal dysfunction-cholestasis and Hermansky-Pudlak (HP) syndromes] are associated with defective vesicle transport, which is an essential process for many cellular events. Now, Neuhauss, Dahm and colleagues identify the zebrafish mutant *leberknödel* (*lbk*) as a model for such disorders (see p. 387). These mutants, like individuals with HP syndrome and similar disorders, have hypopigmented skin melanocytes and a hypopigmented retinal pigment epithelium (RPE). The mutant fish also have visual and immunological defects and defects in various internal organs. The researchers identify the mutation in *lbk* as a loss-of-function mutation in the gene encoding Vam6p/Vps39p, which is required for the trafficking of lysosomes and lysosome-related vesicles, such as melanosomes. They also report that macrophages and cells in the RPE, liver and intestines of *lbk* mutants contain increased numbers of enlarged intracellular vesicles compared with wild-type cells. Overall, these results highlight *vam6* as a novel candidate disease gene and reveal its requirement for normal development for future research to explore.

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Development 135, 237-247 (2008) doi:10.1242/dev.012054

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 β-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, β-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; Stamataki 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).

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Accepted 24 October 2007

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.standford.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 β-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/β 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

axis. Activation of the β-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 β-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 β-catenin lacking amino acids 29-48 and therefore lacking phosphorylation sites necessary for degradation, 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 Tcf3 fused to the repressor domain of Engrailed protein Tcf3^{EnR} or to the VP16 transactivator of herpes simplex virus Tcf^{VP16} (Kim et al., 2000); the sequence encoding amino acids 325-404 of the zebrafish Tcf3 containing the HMG box DNA-binding domain Tcf^{HMG}; a full-length human Gli3, a deleted form of Gli3 encoding amino acids 1-768, Gli3^{R} (Persson et al., 2002); the complementary form encoding amino acids 468-1580, Gli3^{Act} (Stamataki et al., 2005); the sequence encoding amino acids 471-645 of the human Gli3 zinc-finger, Gli^{ZnF} (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 μg/μl in H_2O 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$^{-/-}$; Wnt3a$^{-/-}$ double homozygous mutant embryos

Compound heterozygotes of Wnt1$^{+/-}$ and Wnt3a$^{+/-}$ 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 cryostate (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 β-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 β-catenin^{CA} or Tcf^{DN}, 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 ±s.d. or mean ±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

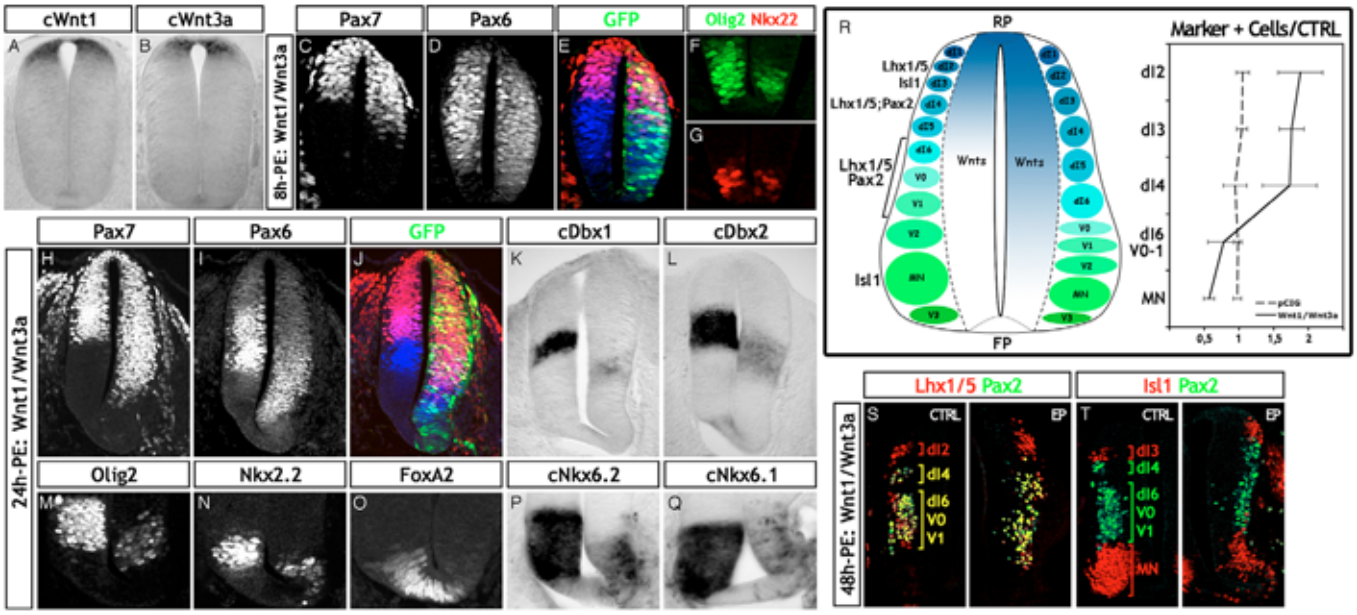

Fig. 1. Dorsally expressed Wnt genes regulate expression of progenitor proteins and result in cell fate changes along the DV axis. (**A**,**B**) HH stage 18 chick embryos show that expression of Wnt1 and Wnt3a is restricted to the dorsal-most neural tube. (**C-G**) Eight hours post-electroporation (8 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 β-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 β-catenin that is resistant to targeted proteolysis [and thus acts as a dominant active protein (β-catenin^{CA}) (Tetsu and McCormick, 1999)]. β-Catenin^{CA} 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 β-catenin^{CA} and assayed 24 hours PE, showed cell-autonomous ectopic activation of dorsal genes such 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

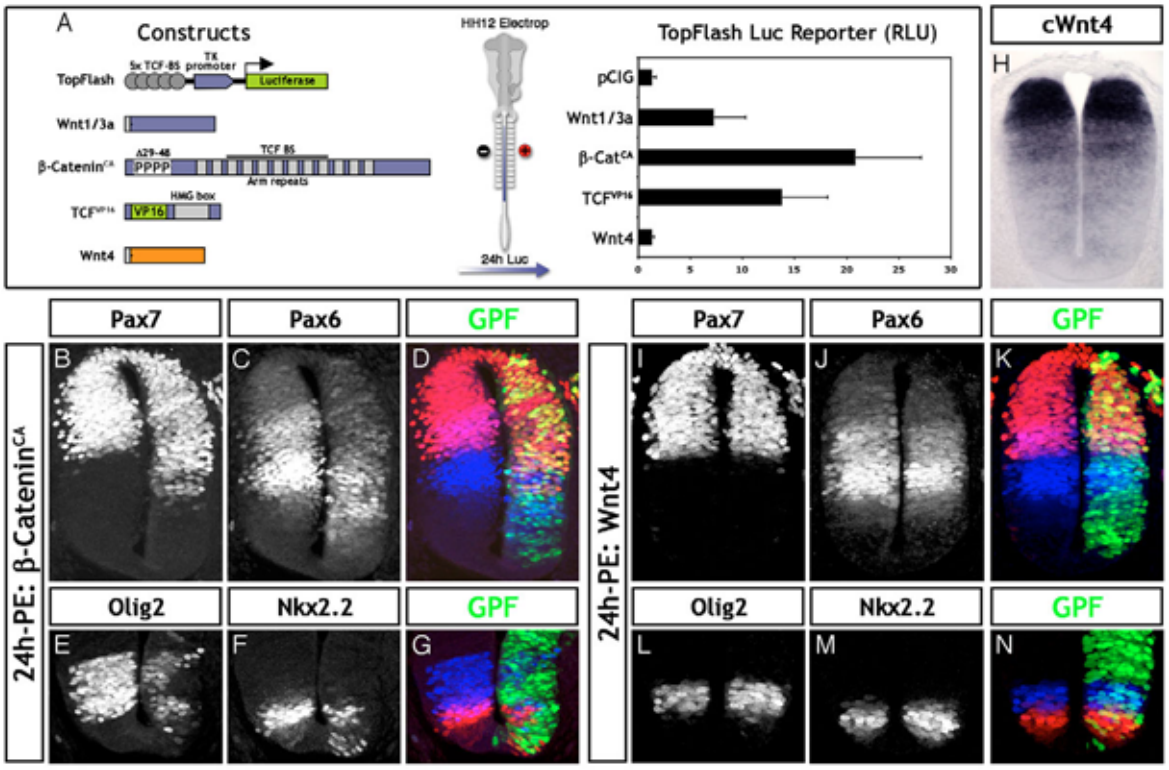

Fig. 2. Wnt patterning activity is mediated by the canonical pathway. (**A**) Activators of the canonical Wnt pathway (left) generate different transcriptional activities in vivo (centre). Embryos were electroporated with Wnt1/3a, β-catenin^{CA}, Tcf^{VP16} and Wnt4, together with the TopFlash reporter 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, β-catenin^{CA} 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 β-catenin-interacting domain, thus acting as constitutive repressors of Wnt target genes (Kim et al., 2000; Tetsu and McCormick, 1999). Tcf1^{DN}, Tcf3^{DN} and Tcf4^{DN} act as strong transcriptional repressors on the TopFlash luciferase reporter (Fig. 3E).

Eight hours PE of any Tcf^{DN} 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^{DN} (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^{DN} resulted in highly comparable ventralized neural tubes (see Fig. S1A-L in the supplementary material), with Tcf4^{DN} 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^{EnR}) that acts as a strong transcriptional repressor on a luciferase assay in ovo (Fig. 3E). Electroporation of Tcf3^{EnR} resulted in a phenotype identical to that of Tcf^{DN} (see Fig. S1M-R in the supplementary material). Converse experiments electroporating the HMG box fused to the transactivator domain of VP16 (Tcf3^{VP16}) 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.

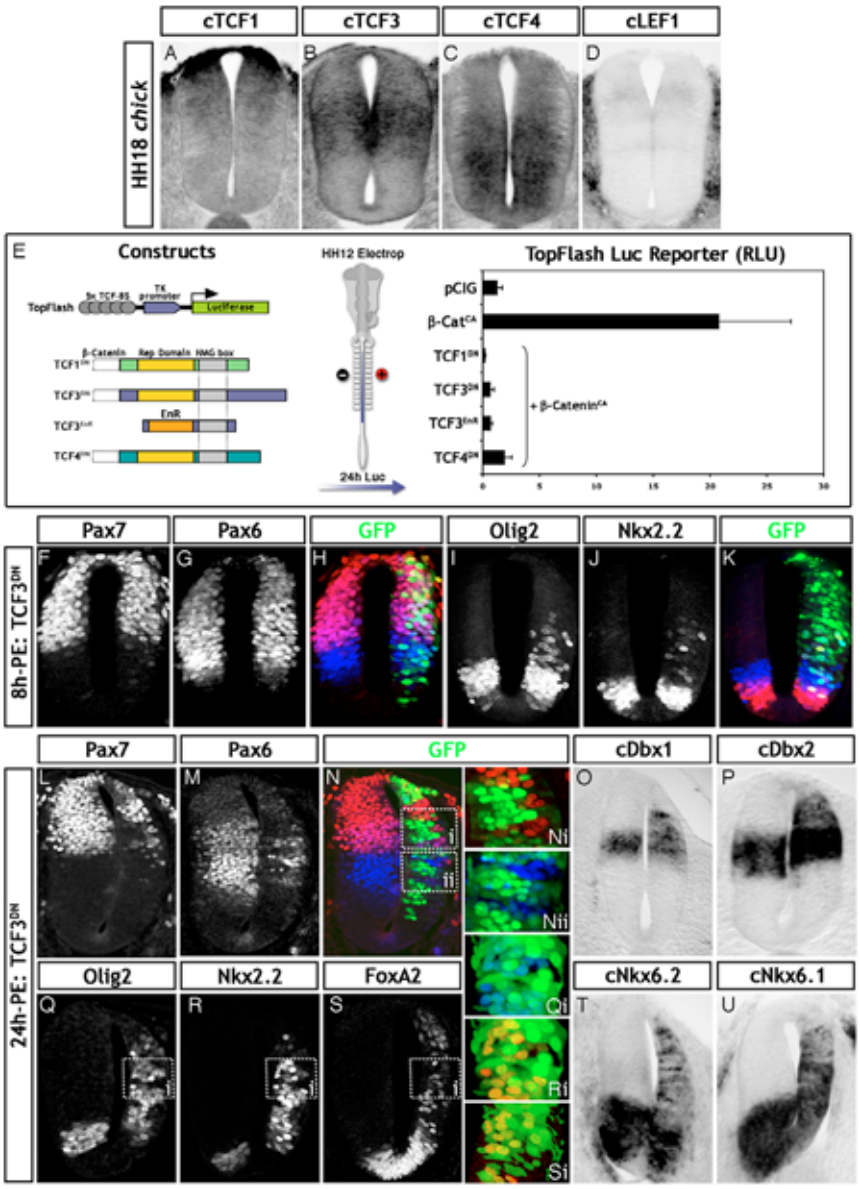

Fig. 3. Tcfs expressed throughout the DV axis regulate patterning of the neural tube. (**A-D**) HH stage 18 embryos showing expression of chick TCF1 (A), chick TCF3 (B), chick TCF4 (C) and chick LEF1 (D). (**E**) In vivo quantitative analysis of the repressors activities of components of the canonical 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 β-catenin-induced luciferase activity. (**F-K**) Eight hours PE of $Tcf3^{DN}$, 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 $Tcf3^{DN}$, 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±16.1% Nkx2.2+ cells and 24.9±13.3% Olig2+ cells 24 hours PE of Wnt1/Wnt3a; 26.70±7.48 Nkx2.2+ cells and 23.45±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

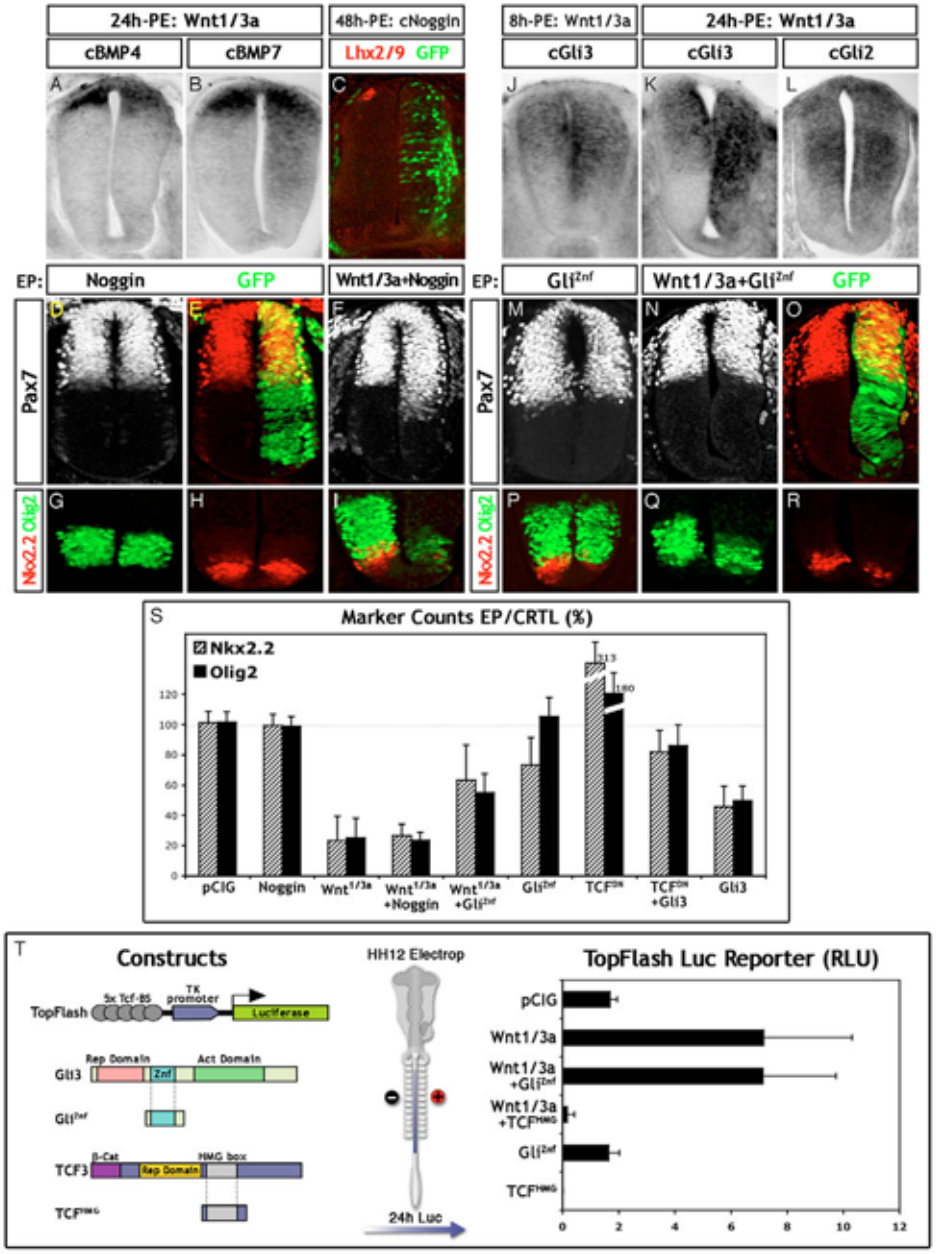

Fig. 4. Wnt activity in DV patterning is independent of Bmp but dependent on Shh/Gli activity. (**A**,**B**) HH stage 11/12 embryos were electroporated with Wnt1 and Wnt3a, and analysed 24 hours PE, for the expression of Bmp4 and Bmp7 by in situ hybridization. (A) Bmp4 expression was weakly expanded 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 dI1. (**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^{ZnF} alone there was no modification of Pax7 expression (N,O) Co-electroporation of Gli^{ZnF} abolished Wnt-induced ventral expansion of Pax7. (O) GFP as a reporter of transgene expression. (P) Twenty-four hours PE, Gli^{ZnF} alone reduced Nkx2.2 (red) expression. (Q,R) Co-electroporation of Wnt1/3a and Gli^{ZnF} 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/β-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^{ZnF} had no positive effect on the TopFlash reporter and cannot repress Wnt-induced luciferase. Electroporation of Tcf^{HMG} 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 (Matise 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^{ZnF}) (Cayuso et al., 2006). As expected Gli^{ZnF} 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^{ZnF} 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^{ZnF} 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^{ZnF} 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 (Litingtung and Chiang, 2000; Stamataki et al., 2005). Furthermore, co-electroporation of Gli^{ZnF} 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^{DN} together with Gli^{ZnF}. Results showed that repression of β-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^{DN} 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^{DN} together with Gli^{ZnF} 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 β-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^{DN} by co-expression of Gli3 with the dominant negative Tcf. Gli3 electroporation reversed the ventralizing activity of Tcf^{DN} [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^{DN}, 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/β-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 $β\text{-catenin}^{CA}$ or with Tcf^{DN} in order to test for their responsiveness to β-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 $β\text{-catenin}^{CA}$ increased R1-R4 transcriptional activity (R2 to ~64 and R3 to ~28 units), while co-electroporation with Tcf^{DN} significantly reduced their activity (R2 to ~6 and R3 to ~2 units), indicating that these modules responded to Wnt/β-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 $Gli3^{Act}$, a strong activator of the Shh pathway (Stamataki 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^{DN} activation was lost by the co-electroporation of either Gli3 or by Tcf^{HMG}, indicating an indirect transcriptional mechanism (Fig. 6A).

Altogether, our results show that the canonical Wnt/β-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).

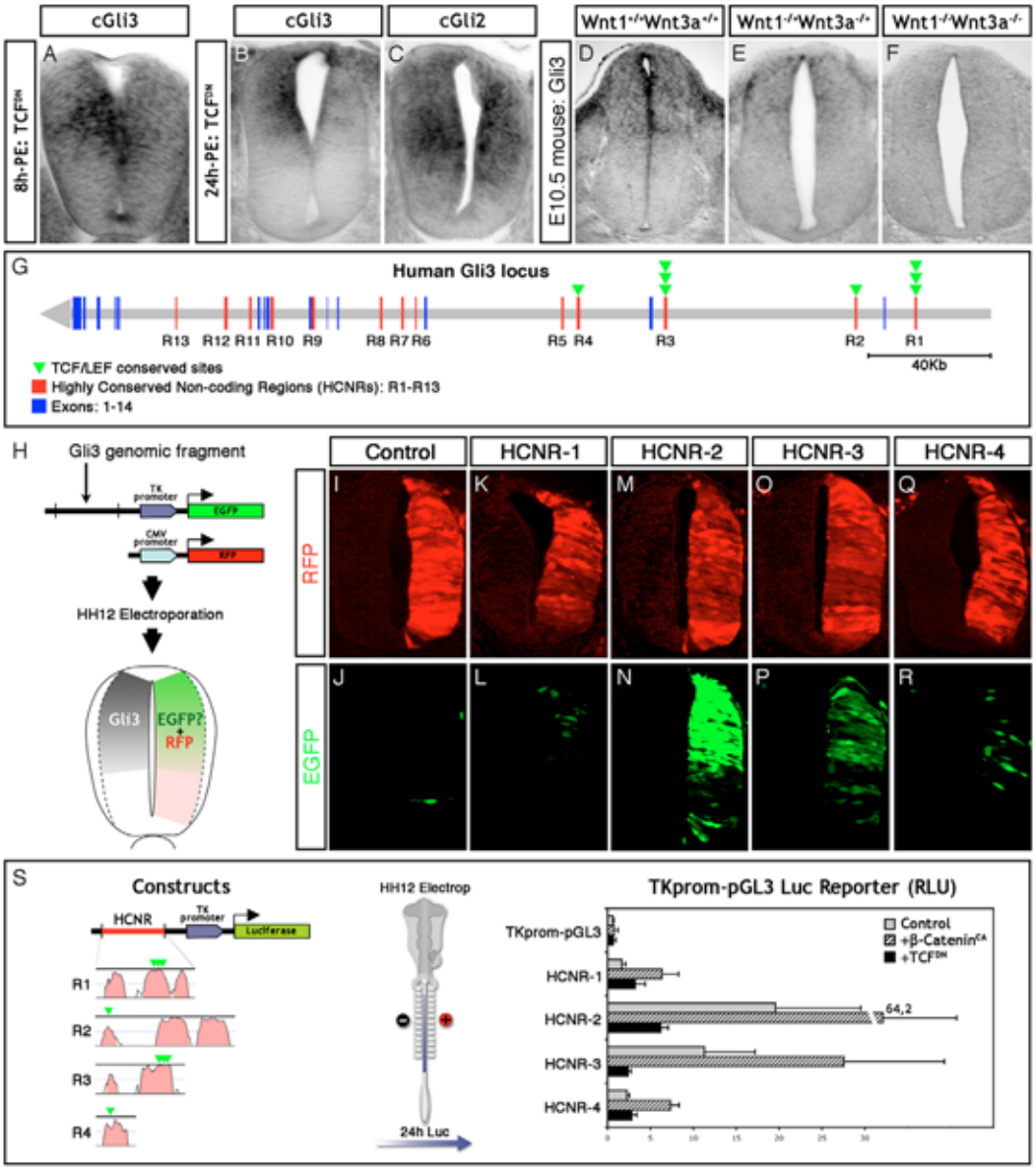

Fig. 5. Wnt signalling controls Gli3 expression in the dorsal spinal cord. (**A-C**) Embryos were electroporated with Tcf^{DN} and analysed for the expression of *Gli3* and *Gli2* by in situ hybridization. (A) Eight hours PE, Tcf^{DN} caused a reduction of Gli3. (B) Twenty-four hours PE, co-electroporation of Tcf^{DN} resulted in the loss of Gli3 expression. (C) Twenty-four hours PE, co-electroporation of Tcf^{DN} 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*$^{+/+}$*; Wnt3a*$^{+/+}$), double heterozygous (*Wnt1*$^{-/+}$*; Wnt3a*$^{-/+}$) and double homozygous (*Wnt1*$^{-/-}$*; Wnt3a*$^{-/-}$) 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-$catenin^{CA}$ or Tcf^{DN}. 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

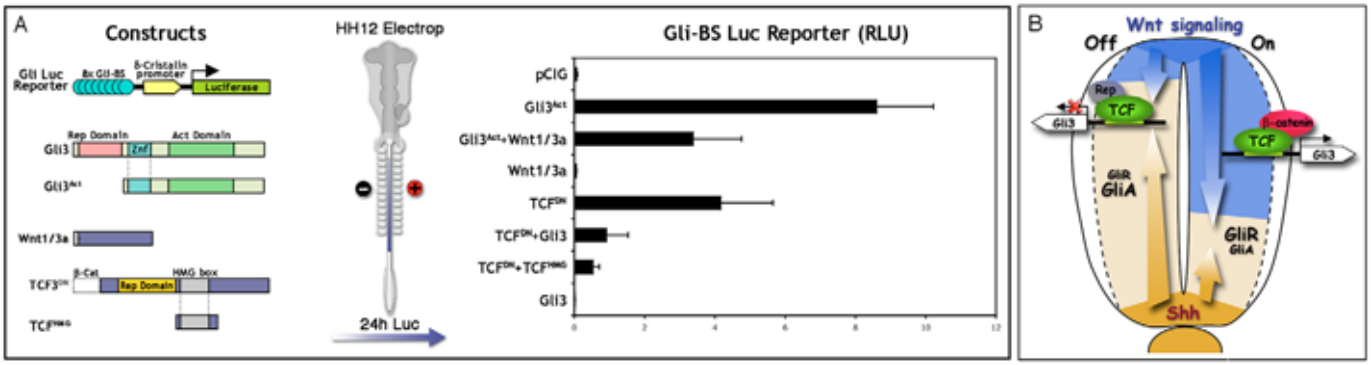

Fig. 6. The Wnt canonical pathway regulates transcriptional Shh/Gli activity. (**A**) In vivo quantitative analysis of the transcriptional activities of several components of the Wnt/β-catenin and the Shh/Gli pathways on a Gli-BS reporter. HH stage 11/12 embryos were electroporated with the indicated DNAs. Embryos were assayed 24 hours PE for luciferase activity. The mutant Gli3 protein, $Gli3^{Act}$, showed high activity on the reporter; this is partially reduced by co-electroporation of Wnt1/Wnt3a. Wnt1/Wnt3a alone had no transcriptional effect on the Gli-BS reporter. Electroporation of Tcf^{DN} caused a potent activation of the reporter, activation that was inhibited by either wild-type or TCF^{HMG} Gli3. (**B**) Schematic representation of a model for dorsoventral patterning of the spinal cord by antagonistic activities of Shh and Wnt signalling pathways.

patterning of the spinal cord, and our data indicate that this role is largely dependent on Gli activity. Moreover, we show that Gli3 expression in the dorsal NT is directly controlled by β-catenin/Tcf, indicating an indirect mechanism generated by Wnt signalling to repress Shh activity in the dorsal NT.

During spinal cord development, Wnt1 and Wnt3a mRNAs are co-expressed at a highly restricted dorsal domain. However, in vivo reporter-gene analysis predicted canonical Wnt signalling to be active in the dorsal two-thirds of the developing mouse (Borello et al., 2006; Maretto et el., 2003) and chick (Megason and McMahon, 2002) spinal cord, suggesting a broader function for Wnt. Our results show that co-electroporation of both genes in developing chick neural tube results in prominent changes in progenitor gene expression along the DV axis. Dorsal genes such as Pax7 and Pax6 are ventrally expanded at the expense of intermediate (Dbx1/2) and ventral (Nkx6.1/6.2, Olig2, Nkx2.2 and Foxa2) gene expression. Expansion of dorsal progenitor gene expression results in the increased generation of dorsal neuronal subtypes (dI1-dI4) with the concomitant loss of ventral motoneurons. Furthermore, electroporation of activated components of the canonical Wnt pathway (β-$catenin^{CA}$ and Tcf^{VP16}) results in the cell-autonomous dorsalization of the NT, indicating that the Wnt activity in DV pattern formation was mediated by the canonical β-catenin/Tcf pathway. Consistent with a role for the Wnt pathway in cell fate specification, it has been shown that Wnt signalling is required for the specification of dorsal cell identities in the mouse (Backman et al., 2005) and the avian (Gunhaga et al., 2003) telencephalon, and in the mouse spinal cord (Muroyama et al., 2002). Additionally, we observed that alterations in cell identities along the DV axis appeared to be accompanied by a prominent growth of the electroporated neural tubes, as previously reported after activation of the canonical Wnt pathway both in mouse and chick developing CNS (Dickinson et al., 1994; Megason and McMahon, 2002; Zechner et al., 2003).

Activation of the canonical Wnt pathway results in activation of the Tcf/Lef family of HMG-box transcription factors. In the nucleus, in the absence of Wnt signal, Tcfs act as repressors of Wnt target genes. β-Catenin can convert Tcf into a transcriptional activator of the same genes that are repressed by Tcf alone (Logan and Nusse, 2004). To test the activity of Tcfs in spinal cord development, we first investigated the expression pattern of members of the family. Tcf1, Tcf3 and Tcf4 are differentially expressed in the developing spinal cord (Schmidt et al., 2004) with their expression domains encompassing the entire DV axis. This suggested a role for Tcf-mediated transcription throughout the developing spinal cord. Electroporation of dominant-negative (DN) forms of Tcf1, Tcf3 and Tcf4 (Tetsu and McCormick, 1999) resulted in highly comparable phenotypes, indicating a redundant function for the three genes in spinal cord development, although some non-redundant tissue-specific responses have been shown for different Tcf/Lef transcription factors in early *Xenopus* development (Liu et al., 2005). Tcf^{DN} caused the cell-autonomous ectopic activation of ventral genes such as Olig2 and Nkx2.2, and the concomitant loss of dorsal genes such as Pax6 and Pax7. All together, results obtained by ectopic activation or repression of the Wnt pathway revealed a prominent role in pattern formation throughout the DV axis, and we propose this to be achieved largely, though not exclusively, by the regulation of Gli3 expression.

Shh signals by binding to its receptor patched 1 (Ptch1), a multi-pass transmembrane protein. In the absence of Shh, Ptch1 acts to suppress the activity of a second transmembrane protein, smoothened (Smo) (for reviews, see Ingham and McMahon, 2001; Lum and Beachy, 2004). Liganding of Ptch1 by Shh relieves repression of Smo, then, through a mechanism yet to be fully elucidated, Smo signals intracellularly to zinc finger-containing transcription factors of the Gli family: highly conserved transcriptional mediators of the Shh pathway that can activate or repress transcription of specific target genes (reviewed by Jacob and Briscoe, 2003). Shh signalling controls cell fates in the developing ventral neural tube, and it has been demonstrated that a gradient of Gli activity is sufficient to mediate, cell-autonomously, the full range of Shh responses (Stamataki et al., 2005). Gli2 and Gli3 are differentially expressed in the developing spinal cord, having some functional redundant and non-redundant roles, with Gli3 repressor activity being required for proper DV patterning (Matise and Joyner, 1999; Jacob and Briscoe, 2003). Thus, regulation of Gli3 expression is a key element in DV patterning.

Our results show that expression of Gli3 within the dorsal NT is directly proportional to Wnt activity, as mice mutant for Wnt1 and Wnt3a (Muroyama et al., 2002) show diminished Gli3 expression. Gain and loss of β-catenin/Tcf function in chick embryos also directly regulates Gli3 expression. Furthermore we characterized

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 β-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/β-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 β-catenin provides an attractive working model (Ulloa et al., 2007).

In addition, prominent signalling molecules resident in the roof plate are members of the Tgfβ/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 Tgfβ/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, Tgfβ/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).

We thank Susana Usieto for invaluable research assistance and Dr J. L. Gomez-Skarmeta for help with the analysis of genomic GLI3. Monoclonal antibodies were all obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. We also thank Drs A. P. McMahon, J. Briscoe, E. Sancho, E. Batlle, A. Kispert, A. Munsternberg, D. Rowitch and H. Kondoh for DNAs and reagents. Work in the E.M.'s laboratory is supported by the Spanish Ministry of Education Grant BFU2004-00455/BMC and Fundación La Caixa BM05-52-0, that also supported J.C. R.A.M. is supported by a FI predoctoral fellowship from Generalitat de Cataluña.

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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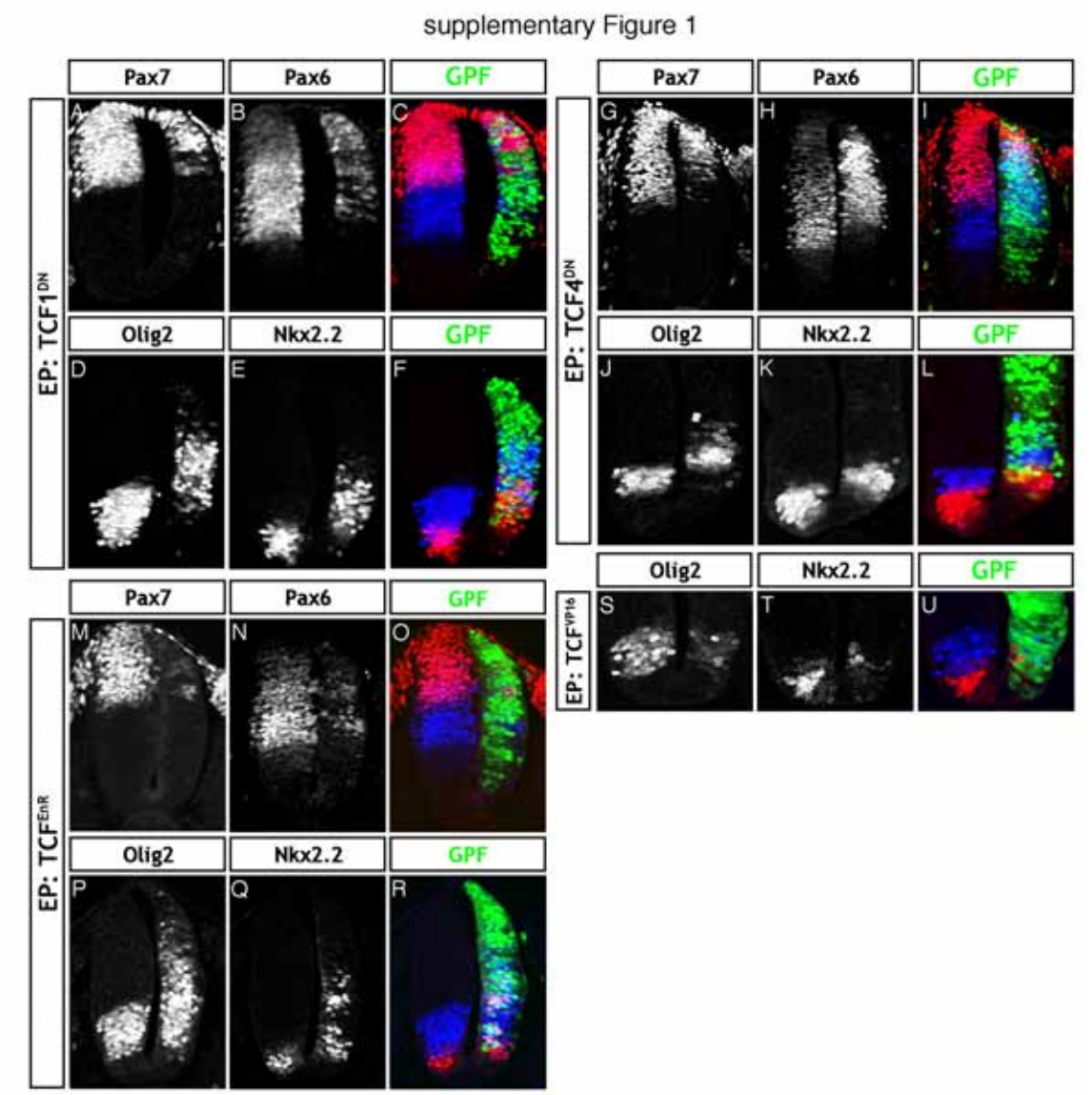
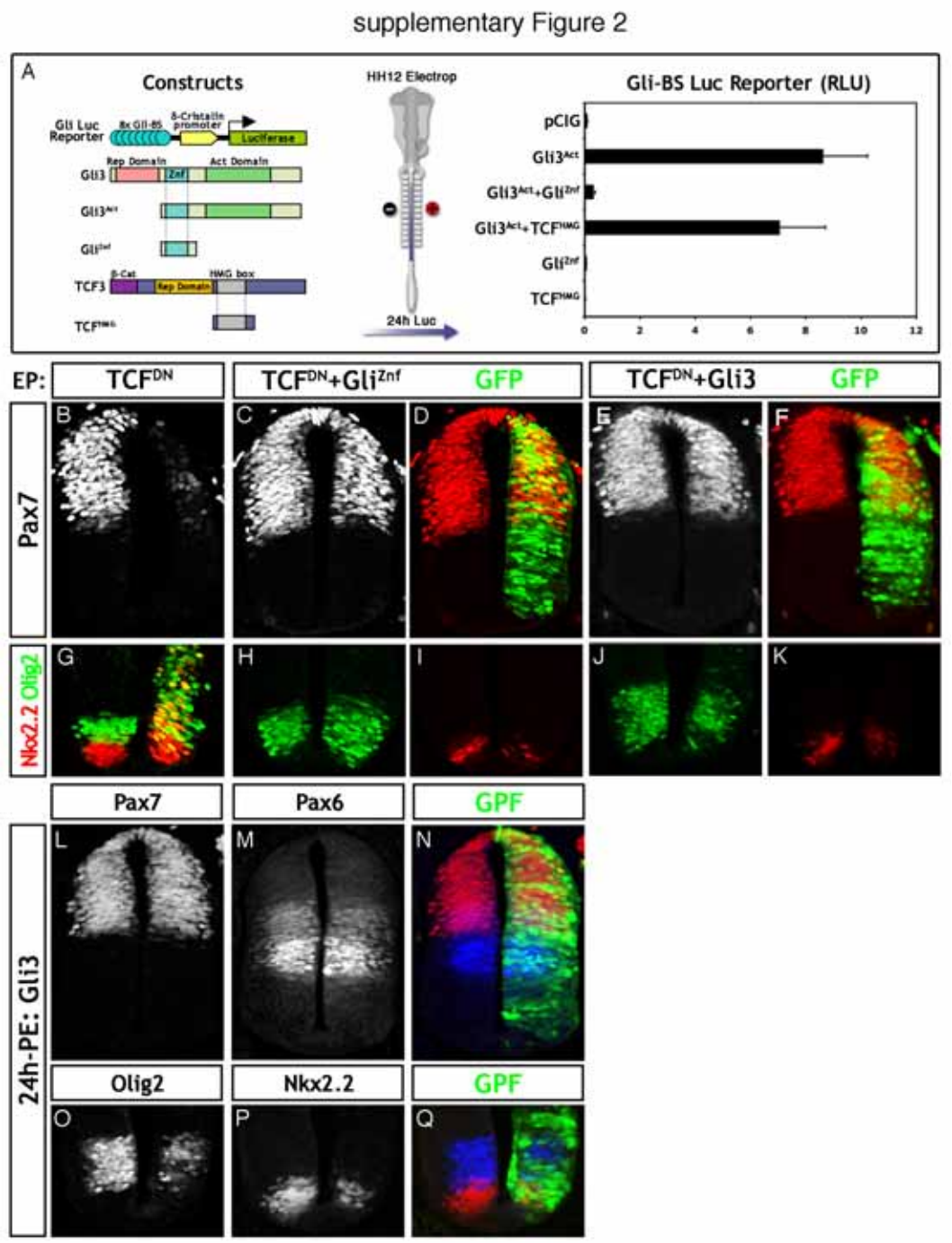
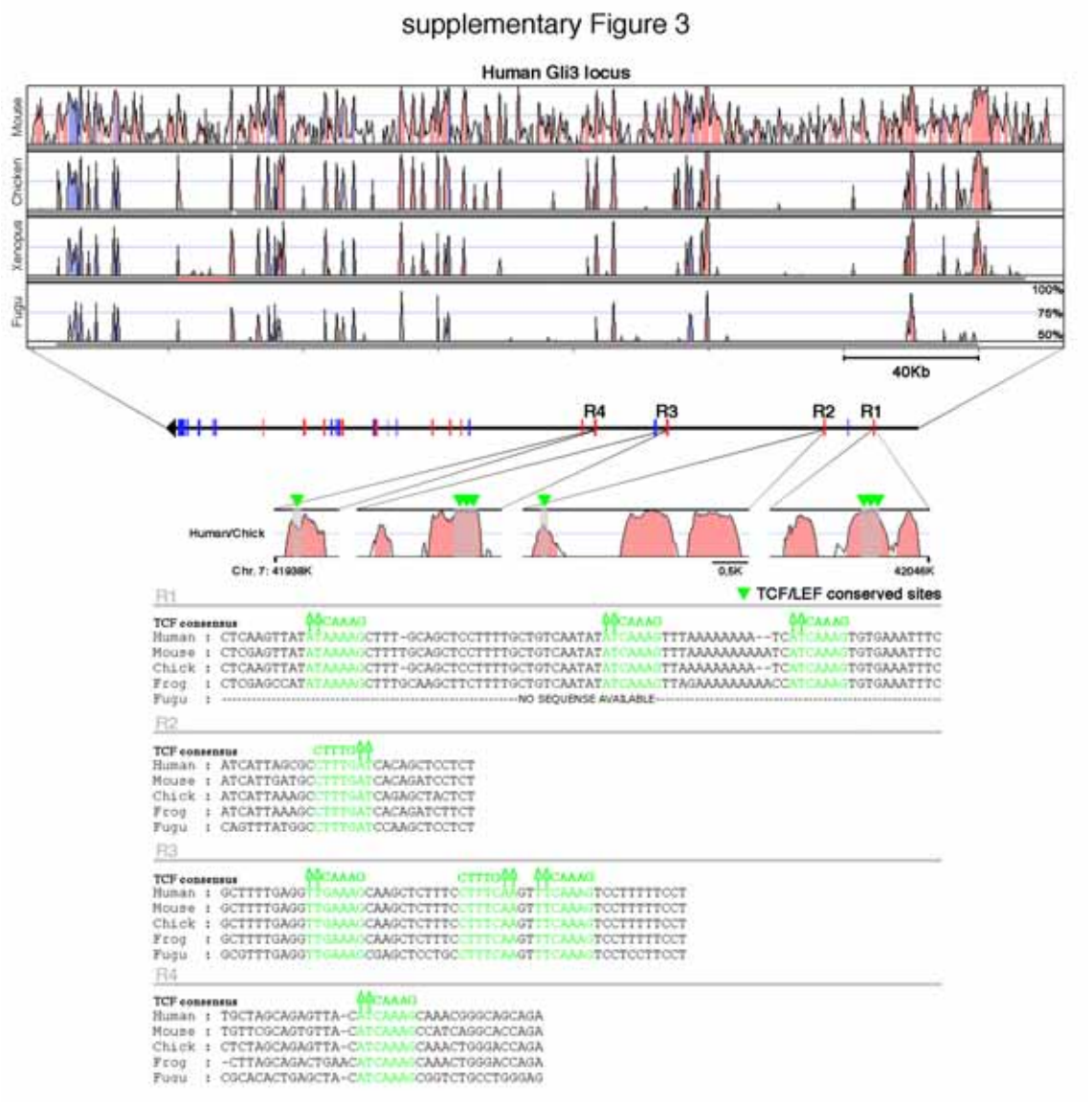


SUPPLEMENTARY FIGURE 1. Different TCF genes have similar patterning activities in the neural tube. HH stage 11/12 embryos were electroporated with the indicated DNAs, and analysed twenty four hours after electroporation (24h-PE) for progenitor gene expression. (A-F) Electroporation of TCF1^{DN} reduced expression of dorsal progenitor proteins Pax7 (A) and Pax6 (B). GFP was used as a reporter of transgene expression (C). Ventral genes Olig2 (D), Nkx2.2 (E) were ectopically and cell autonomously activated in electroporated GFP-expressing cells (F). (G-L) Electroporation of TCF4^{DN}, resulted in similar changes in progenitor gene expression. (M-R) Electroporation of TCF3 fused to the repressor domain of Engrailed (TCF3^{EnR}) resulted in similar changes in progenitor gene expression. (S-U) Electroporation of TCF3 fused to the VP16 activator domain (TCF3^{VP16}) resulted in the opposite phenotype, the cell autonomous reduction of ventral gene expression.



SUPPLEMENTARY FIGURE 2: Wnt patterning activity depends on Gli. (A) HH stage 11/12 embryos were electroporated with the indicated DNAs, diagram shows schematic representation of DNAs, together with the Gli-BS reporter containing 8Gli-binding sites. Embryos were assayed 24h-PE for luciferase activity. $Gli3^{Act}$ showed high positive activity on the Gli-BS reporter, transcriptional activity that is repressed by the Gli^{ZnF} but not by the TCF^{HMG}. Gli^{ZnF} and TCF^{HMG} alone showed no positive activity on the Gli-BS reporter. (B-F) TCF mediated loss of Pax7 was dependent on Gli activity. (B) 24h-PE, TCF^{DN} alone caused loss of Pax7 expression. (C,D) Co-electroporation of TCF^{DN} together with Gli^{ZnF} totally rescued Pax7 expression. (E,F) Co-electroporation with TCF^{HMG} also rescued Pax7 expression. (G-P) Co-electroporation experiments showed partial rescue of ventral gene expression. (G) 24h-PE, TCF^{DN} alone induce ectopic Olig2 (green) and Nkx2.2 (red) expressing cells. (H,I) Co-electroporation of TCF^{DN} and Gli^{ZnF} resulted in the rescue of Olig2 and Nkx2.2 expression, although few ectopic Olig2+ cells still appeared. (J,K) Co-electroporation of TCF^{DN} and TCF^{HMG} resulted in the rescue of Olig2 and Nkx2.2 expression, although few ectopic Nkx2.2+ cells still appeared. (L-Q) Twenty four hours after electroporation (24h-PE) of $TCF3^{HMG}$, expression of dorsal progenitor proteins Pax7 (L) and Pax6 (M) was not modified. GFP as a reporter of transgene expression (N). Expression of ventral Olig2 (O) and Nkx2.2 (P) was reduced in cells ex-



SUPPLEMENTARY FIGURE 3: Analysis of the human *Gli3* locus and characterization of HCNRs. Sequence alignments of the genomic interval containing the human *GLI3* locus, with orthologous counterparts from representative members of the rodents, birds, amphibian and fish lineages. Conserved coding sequences are depicted in blue and conserved non-coding sequences in pink. Arrow shows the length of the *GLI3* gene and the direction of transcription. TCF binding sites are depicted in green. Identification of consensus TCF binding sites within the human *GLI3* locus. Comparison of HCNR1-R4 sequences from the human locus with other vertebrates. All vertebrate species examined contained the core consensus TCF biding site (green). FUGU sequence is not annotated on region R1, therefore comparison does not appear in the figure.

La regulación de la proliferación en los progenitores neuronales del tubo neural requiere la integración de las actividades Wnt y Shh

Las vías canónicas de señalización celular de Wnt y Hedgehog regulan la proliferación celular en varios contextos de forma conjunta, Sin embargo, posibles interacciones de estas vías de señalización en el control del ciclo celular no han siso estudiadas. Durante el desarrollo del sistema nervioso de vertebrados, aunque las proteínas Shh y Wnts se expresan en extremos dorso-ventralmente opuestos del tubo neural, varios trabajos demuestran que la proliferación de los progenitores neuronales requiere ambas actividades a lo largo de todo el eje dorsoventral. En este trabajo, demostramos que es necesaria la integración de ambas vías de señalización para controlar la duración de la fase G1 del ciclo celular. Además, mostramos el requerimiento de la actividad Hedgehog para la regulación mediada por Wnt de la expresión del activador de ciclo celular ciclina D1, componente clave para la progresión a través de G1. Aunque la actividad de la vía canónica de Wnt se limita al control de la transición G1/S, adicionalmente, la actividad Hedgehog regula la duración de la fase G2 mediante la regulación de las ciclinas de fase G2 ciclina A2 y Ciclinas B2/B3. En conjunto, estos resultados proponen un papel fundamental en el control del crecimiento para la actividad Shh/Gli como reguladora de las fases G1 y G2 del ciclo celular y además como reguladora por encima de la actividad canónica de Wnt.

Control of neuroepithelial cell cycle progression requires integration of Wnt and Sonic hedgehog activities

Control of neuroepithelial cell cycle progression requires integration of Wnt and Sonic hedgehog activities

Roberto Alvarez-Medina and Elisa Martí*

Summary

The Wnt canonical pathway and Hedgehog signalling have been linked to cell proliferation in a variety of systems, however interaction of these pathways to control cell cycle progression have not been studied. In the developing vertebrate nervous system, although Shh and Wnt ligands are expressed at the opposite ventral and dorsal signalling centres, reports demonstrate that proliferation of neural progenitors require both activities throughout the dorsoventral axis. Here we demonstrate the integration of both pathways to control the length of G1 phase, and the absolute requirement of an upstream Hedgehog activity for the Wnt-mediated regulation of the key cell cycle activator CyclinD1 expression and for G1 progression. Although Wnt canonical activity appeared restricted to the control of G1 phase, Hedgehog activity additionally regulates the length of G2 phase through the regulation of late cell cycle activators such as CyclinA2 and CyclinB2/3. These findings support a key role for Hedgehog in growth control, as a regulator of G1 and G2 phases of cell cycle and importantly as an upstream regulator of the canonical Wnt activity.

Key words: Neural development, spinal cord, chick embryo, cell cycle progression, proliferation control, Sonic hedgehog signalling, Wnt canonical pathway, Gli proteins, b**-Catenin, TCF transcription factors**

INTRODUCTION

The early embryonic vertebrate neural tube is composed of proliferating progenitors and terminally differentiating neurons distributed in a characteristic arrangement. Mitotically active cells form a pseudostratified epithelium that occupies the medially located ventricular zone, as cells differentiate in this progenitor region they exit the cell cycle and migrate in a medial to lateral direction to form a mantle zone of post-mitotic neurons (Ramón y Cajal, 1955). Distinct neuronal subtypes emerge in a precise spatial order from progenitor cells resulting in the partition of the dorsal-ventral axis of the neural tube into discrete regions occupied by different neuronal subtypes (Jessell, 2000). At the same time, a balance between differentiation and proliferation of progenitors ensures the growth of the neural tube and replenishes the pool of progenitors allowing continued rounds of neuronal differentiation. Signals that control proliferation in the neural tissue are not well understood. Although a number of studies suggested an important role for the Wnt canonical pathway, increasing experimental data supports that Sonic hedgehog (Shh) activity also play a major role, raising the question as to whether these two activities act in parallel or whether they integrate to regulate proliferation of neural progenitors.

Wnts are a large family of highly conserved secreted signalling proteins related to the Drosophila wingless protein that regulates cell-to-cell interactions during embryogenesis (Nusse, 2004). Evidence that Wnt proteins regulate of growth in multiple areas of the CNS come from analysis of mouse mutants for Wnt1 (McMahon and Bradley, 1990; Thomas and Cappechi, 1990), Wnt3a (Lee et al., 2000), and Wnt1/Wnt3a (Ikeya et al., 1997; Muroyama et al., 2002) that have reduced midbrain, hippocampus, caudal diencephalon, rostral hindbrain or spinal cord, depending on the mutations. Consistent with a prominent role in precursor cell

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Article in progress 2008

proliferation, ectopic expression of Wnt1 or Wnt3a causes overgrowth of the neural tube (Dickinson et al., 1994; Megason and McMahon, 2002; Panhuysen et al., 2004). Evidence that the Wnt-dependent control of proliferation is mediated through the canonical Wnt-pathway 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 cerebral cortex and hippocampus, is reduced after ablation of β-catenin and that the neural precursor population is not maintained. In contrast, the spinal cord of chick, and the brain of mice that express activated β-catenin have an enlarged mass with an increased population of neuronal precursors (Megason and McMahon, 2002; Zechner et al., 2003).

Sonic hedgehog (Shh) is one of the members of the hedgehog family of secreted proteins required for multiple aspects of development in a wide range of tissues including the CNS (reviewed in McMahon et al 2003). Supporting a role for the Shh pathway in the control of neural progenitor proliferation, blockade of Shh signaling either by the genetic removal of Shh or components of the Shh pathway (Chiang et al., 1996; Litingtung and Chiang, 2000; Wijgerde et al., 2002; Cayuso et al., 2006) results in reduced neural tissue. Converserly, ectopic expression of Shh (Rowitch et al., 1999) or ectopic activation of the Shh signaling pathway (Goodrich et al., 1997; Epstein et al., 1996; Hynes et al., 2000; Cayuso et al., 2006) results in hyper-proliferation of progenitors. Furthermore, genetic manipulation of the dosage of two endogenous inhibitors of the pathway, Patched1 and Hip1, results in a noticeable and dose-dependent enlargement of the neural tube (Jeong and McMahon, 2005).

Opposite expression of Wnt and Shh ligands; Wnts largely dorsal and Shh largely restricted to the floor plate cells of the ventral most CNS, suggested a simplistic model in which these two pathways might act in parallel on different precursor populations, i.e Wnts-dorsal and Shh-ventral? Alternative models include 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 cell cycle regulators.

Two key regulators of the G1/S transition of cell cycle are targets of the Wnt canonical pathway: cyclin D1 (Shtutman et al., 1999; Tetsu and McCormick, 1999) and c-Myc (He et al., 1998). Transcriptional activation of cyclin D1 through the β-catenin/TCF pathway also occurs in the developing neural tube, suggesting that Wnt signalling positively regulates cell cycle progression and negatively regulates cell cycle exit of spinal cord precursors, in part through transcriptional regulation of cyclin D1 (Megason and McMahon, 2002; Panhuysen et al., 2004). Additionally, expression of N-Myc and cyclinD1 is also regulated by the mitogenic activity of Shh, both in the developing cerebellum (Oliver et al., 2003; Kenney and Rowitch, 2000; Kenney et al., 2003) and the early neural tube (Ishibashi and McMahon, 2002, Lobjois et al., 2004; Cayuso et al., 2006). Further, conditional knockout of N-Myc in neuronal progenitor cells results in a dramatic reduction in brain mass suggesting that N-Myc is required for the rapid expansion of progenitor cell populations (Knoepfler et al., 2002).

Here, we show members of the TCF family of transcription factors expressed in the neural tube to control cell cycle progression through G1 phase checkpoint. Inhibition of the Shh and Wnt/β-catenin pathways at the membrane level shows that both signals are required for cell cycle progression along the DV axis of the neural tube. Epistatic experiments demostrate that Wnt mitogenic activity and cyclinD1 expression is dependent on a Shh/Gli activity. Moreover, while Wnt regulation of cell cycle progression appears to be restricted to G1, Shh activity extends through G2 phase of cell cycle, regulating the expression of G2 cyclins A2, B2 and B3. Altogether, our results indicate that Shh/Gli activity, by an indirect transcriptional mechanism, is modulating Wnt mediated G1 cell cycle progression and, independently, G2 phase of the cell cycle.

MATERIALS AND METHODS

DNA Constructs

The following DNAs were inserted into pCIG, upstream of an internal ribosomal entry site (IRES) and three nuclear localization sequences tagged EGFP in

pCAGGS expression vector (Megason and McMahon, 2002); full coding mouse Wnt1, Wnt3a (Megason and McMahon, 2002); a mutant form of β-catenin lacking amino acids 29-48 and therefore lacking phosphorylation sites necessary for degradation, β-catenin^{CA} (Tetsu and McCormick, 1999); a mutant version of the LDL-related protein LRP6 lacking the citoplasmic domain (LRP6^{DC}) and thus acting as a dominant negative co-receptor for the Wnt pathway (Tamal et al., 2000); 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 TCF3 fused to the repressor domain of Engrailed protein TCF3^{EnR} or to the VP16 transactivator of herpes simplex virus TCF3^{VP16} (Kim et al., 2000); a deleted form of mouse patched-1 lacking the second large extracellular loop (Ptc$^{\Delta Loop}$) (Briscoe et al., 2001); a deleted form of human Gli3 encoding amino acids 1-768, Gli3^{R} (Persson et al., 2002); the complementary form encoding aminoacids 468-1580, Gli3^{Act} (Stamataki et al., 2005); the sequence encoding amino acids 471-645 of the human Gli3 zinc-finger, Gli^{Znf} (Cayuso et al., 2006); and full length Cyclin D1 (Lobjois et al., 2008).

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 and Hamilton, 1951).

Chick embryos were electroporated with Clontech purified plasmid DNA at 2-3 µg/µl in H_2O with 50ng/ml Fast Green. Briefly, plasmid DNA was injected into the lumen of HH Stage 11-12 neural tubes, electrodes were placed either side of the neural tube and electroporation carried out using an Intracel Dual Pulse (TSS10) electroporator delivering five 50millisecond square pulses of 30-40 V.

Transfected embryos were allowed to develop to the specific stages, then dissected, fixed and processed for immunohistochemistry, in situ hybridization, or FACS analysis.

Immunohistochemistry

Embryos were fixed 2-4 hours at 4ºC in 4% paraformaldehyde in PB, rinsed, sunk in 30% sucrose solution, embedded in OCT and sectioned in a Leica cryostate (CM 1900). Alternatively, embryos were sectioned in a Leica vibratome (VT 1000S). Immunostainings were performed following standard procedures.

For BrdU detection, sections were incubated in 2N HCl for 30 minutes followed by 0.1M $Na_2B_4O_7$ pH8.5 rinses further PBT rinses and anti-BrdU incubation.

Antibodies against the following proteins were used; green fluorescence protein (GFP) (Molecular Probes), anti-myc (9E10, Santa Cruz). 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 were made on 10-40 different sections of at least 4 different embryos after each experimental condition (n>4).

In situ hybridization

Embryos were fixed overnight at 4ºC in 4% paraformaldehyde in PB, rinsed and processed for whole mount RNA in situ hybridization following standard procedures using probes for chick *TCF1, TCF3, TCF4 and Lef1*, *CyclinD1, CyclinB2, CyclinB3, CylcinA2* (form the chicken EST project, UK-HGMP RC). 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.

Fluorescence Associated Cell Sorting (FACS)

EGFP-containing plasmid DNA was injected into the lumen of HH Stage 11-12 neural tube, embryos were electroporated as described above, and neural tubes dissected out 12-24 hours later. Single cell suspension was obtained by 10-15 minutes incubation on Tripsin-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, Colorado, USA). Excitation of the sample was done using a Coherent Enterprise II argon-ion laser. Excitation with the blue

line of the laser (488nm) permits the acquisition of forward scatter (FS), side scatter (SS) and green (530 nm) fluorescence from GFP. UV emission (40mW) was used to excite Hoescht blue fluorescence (450nm). Doublets were discriminated using an integral /peak dotplot of Hoechst fluorescence. Optical alignment was based on optimized signal from 10 µm fluorescent beads (Flowcheck, Coulter Corporation, Miami, Florida, USA). DNA analysis (Ploidy analysis) on single fluorescence histograms was done using Multicycle software (Phoenix Flow Systems, San Diego, CA).

GFP Transcription Assay

The TOP-GFP and FOP-GFP constructs were used in order to in vivo monitor the extent of βCatenin-TCF activity in the developing chick spinal cord (Sasai et al., 2005). Embryos were electroporated at HH stage 11/12 and GFP expression was examined after 24-48 h. DsRFP gene driven by chicken β-actin promoter (pCAGGS-IRES-DsRFP, M. B-F. unpublished) was co-electroporated as control for transfection efficiency.

Statistical Analysis

Quantitative data were expressed as mean ±s.d. or mean ±s.e.m. Significant differences among groups were tested by Student´s *t*-test.

RESULTS

Wnt canonical activity is required for proliferation throughout the DV axis of the neural tube, and regulates progression of G1 phase of the cell cycle.

Wnt1 and Wnt3a, the most dorsally expressed members of the Wnt family regulate proliferation of spinal cord progenitors acting through the canonical β-catenin/ TCF pathway. To begin to investigate whether TCF activity might regulate proliferation through the DV axis of the developing neural tube (NT), HH staged 12 chick embryos were hybridized with probes to TCF1, TCF3, TCF4 and LEF1. Expression analysis revealed members of the TCF family of transcription factors to be expressed in discrete and partially overlapping expression patterns; TCF1 mainly dorsal (Fig. 1A), TCF3 intermediate (Fig. 1B), and TCF4 mainly in the ventral NT (Fig. 1C), while LEF1 was only weakly expressed in the NT (Fig. 1D). Thus expression analysis suggested a theoretical capacity to generate TCF-mediated transcriptional response throughout the DV axis of the developing NT.

To monitor the spatial activation of the Wnt canonical pathway, we transfected a TCF reporter that uses GFP as reporter gene (TOP-EGFP) (Sasai et al., 2005), together with pCAGGS-DsRFP for electroporation control. A control FOP-GFP that lacks TCF binding sites showed no GFP expression (Fig. 1E), however electroporation of the TOP-GFP construct showed that endogenous Wnt activity has the capacity to active reporter GPF expression through the DV axis of the developing NT (Fig. 1F), thus suggesting the Wnt pathway to be broadly active.

To investigate whether TCF activity is required along the DV axis, embryos were electroporated with dominant negative (DN) forms of TCF1, TCF3 and TCF4, that cannot interact with β-catenin acting as constitutive repressors of Wnt target genes, and as repressors of Wnt/βcatenin transcriptional activity (Alvarez-Medina et al., 2008; Kim et al., 2000). To allow informative comparisons between embryos and constructs, we standardized the conditions used in all experiments: HH 11/12 embryos were electroporated with each construct and following incubation and processing, analysis was restricted to the forelimb and the anterior thoracic regions. Transfected cells, marked by GFP, do not incorporate Bromodeoxyuridine (BrdU) after a 30 minutes pulse, whereas incorporation was observed in neighbouring non-transfected cells and in cells transfected with the control pCIG vector, showing that TCF^{DN} cell-autonomously block entry into the S-phase of the cell cycle, (Fig. 1G-I). Cell counting in dorsal and ventral halves of the NT showed no significant differences in any of the experimental manipulations (Fig. 1J, Table S1), therefore dorsal and ventral data are shown pooled in the rest of the figures.

Repression of TCF target genes by transfection of either $TCF1^{DN}$, $TCF3^{DN}$ or $TCF4^{DN}$ caused a reduction of $BrdU^{+}$ cells to ~25%, ~20%, or ~19% respectively, compared to control embryos (~42% BrdU+ cells 24hours-Post Electroporation of empty pCIG vector)

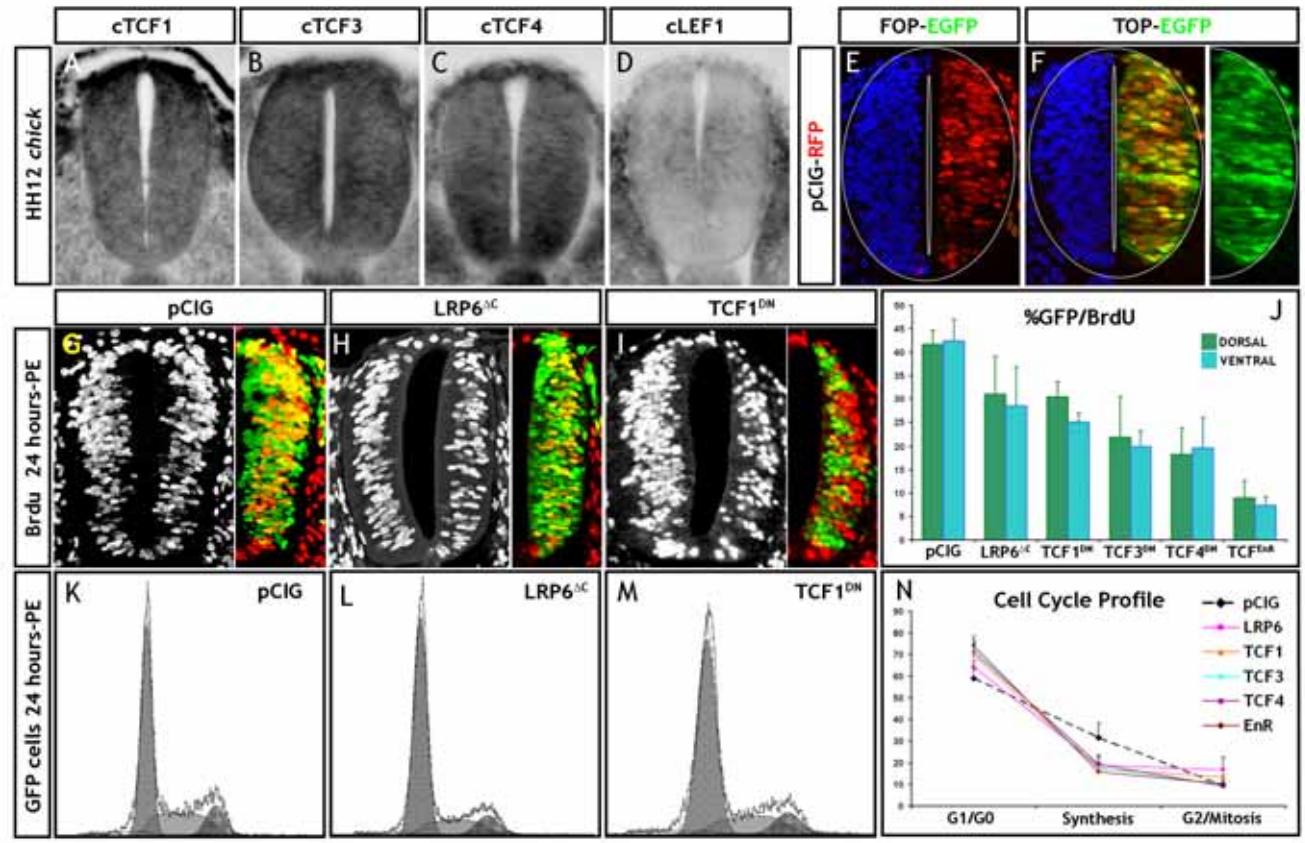

Figure 1. Wnt canonical activity is required for proliferation throughout the DV axis of the neural tube. (A-D) Expression analysis of members of the TCF family of transcription factors. HH12/14 chick embryos were hybridized with probes to TCF1 (A), TCF3 (B), TCF4 (C) and LEF1 (D). (E,F) In vivo monitoring of Wnt canonical activity by electroporation the control FOP-EGFP, or the reporter TOP-EGFP, together with pCIG- DsRed for electroporation control. (G-J) Analysis of proliferation by BrdU incorporation (red) 24h-PE of indicated DNAs (GFP, green), at HH stage 11/12. (G) Control section after pCIG electroporation. (H) Loss-of endogenous Wnt activity by electroporation of a dominant negative version of the co-receptor LRP6 ($LRP6^{\Delta C}$). (I) Loss-of Wnt target genes activation by electroporation of a constitutive transcriptional repressor version of TCF1 (TCF^{DN}). (J) Quantitative analysis of GFP-expressing cells (green) that have incorporated BrdU (red), after 30 minutes BrdU pulse. Analysis was carried out by separating dorsal and ventral halves of the neural tube, 24 hours post electroporation (h-PE) of the indicated DNAs
(K-N) Flow cytometry analysis of cell cycle phase distribution of transfected cells. HH11/12 stage embryos were electroporated with the indicated DNAs. 24h-PE neural tubes were dissected out, GFP-expressing cells separated by flow cytometry and cell cycle phases analysed by Hoescht staining. (K-M) Representative examples of cell cycle profile of cells expressing pCIG (K), $LRP6^{\Delta C}$ (L) and $TCF1^{DN}$ (M). (N) Quantitative analysis of cell cycle phase distribution after electroporation of the indicated DNAs. Loss of Wnt activity resulted in increased percentage of cells arrested at the G1/G0 phase of cell cycle, as compared to control pCIG cells.

(Fig. 1J, Table S1). Furthermore, electroporation of the HMG box DNA-binding domain of TCF3 fused to the repressor domain of Engrailed protein ($TCF3^{EnR}$), resulted in a more dramatic loss of BrdU incorporation (only ~8% BrdU+cells 24h-PE) indicating that blockade of cell cycle progression was indeed the result of repression of Wnt targets. Additionally, electroporation of a dominant negative form of the co-receptor LRP6 ($LRP6^{DN,}$ Tamal et al., 2000), that inhibits endogenous Wnt canonical pathway resulted in the cell autonomous reduction of BrdU+ cells to ~28% (Fig. 1H, J), thus showing the requirement of endogenous Wnt proteins for cell cycle progression both in the ventral and the dorsal NT.

To gain further insights into which phase of the cell cycle are neural cells being arrested, embryos were dissected 24 hours after electropopration (h-PE) and single cell suspensions processed to flow cytometry. Analysis of cell cycle phase distribution, obtained by plotting hoescht-fluorescence intensity versus the number of GFP positive cells, showed transfected cells being arrested at the G1/G0 phase of the cell cycle. Control cells electroporated with the empty pCIG vector were distributed as ~58% cells in the G1/G0 phase, 9,5% cells in the G2 and 31,5% cells in the S-phase of the cell cycle. Electroporation of $TCF1^{DN}$, $TCF3^{DN}$ or $TCF4^{DN}$ increased the percentage of cells in the G1/G0 phase to 69%, 72%, and 71% respectively,

while electroporation of the TCF3^{EnR} construct resulted in 74% cells arrested in the G1/G0 phase (Fig. 1K-N; Table S1). These data indicated that repression of Wnt target genes lengths the G1/G0 phase of the cell cycle in a similar way to what we have previously observed for the repression of Gli target genes (Cayuso et al., 2006), thus suggesting that both pathways might interact to control progression of the G1 phase of cell cycle.

Furthermore, the fact that both Wnt and Shh activities are required throughout the DV axis argue against a simplistic model in which dorsally expressed Wnts control proliferation of dorsal progenitors, while ventrally expressed Shh regulate 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 activity is required for the Wnt regulation of cell cycle progression and expression of CyclinD1.

Shh signals by binding to its receptor Patched (Ptc), a multi-pass transmembrane protein. In the absence of Shh, Ptc acts to suppress the activity of a second transmembrane protein, Smoothened (Smo) (for review see Lum and Beachy, 2004). Inhibition of Shh signaling at the reception level was accomplished by electroporation of a mutant form of the Hh receptor Patched1 (mPtc1$^{\Delta loop2}$) that lacks most of the second large extracellular loop and has lost the capacity to bind Shh but retains the ability to inhibit Smoothened or a downstream Smoothened effector (Briscoe et al., 2001). This resulted in a cell autonomous reduction to ~22% BrdU^{+} cells compared to control embryos electroporated with empty pCIG vector (Fig. 2A, D; Cayuso et al., 2006).

To begin to investigate for a possible genetic interaction between the Wnt and the Shh pathways in the regulation of progenitor cell proliferation, we activated the canonical Wnt pathway in a loss-of-function background for Shh activity. Co-electroporation of mPtc1$^{\Delta loop2}$ with the proliferative ligands Wnt1/Wnt3a (Megason and McMahon, 2002) or with a stabilized form of β-catenin that acts as a dominant active protein (Tetsu and McCormick, 1999), resulted in reduced BrdU incorporation to ~27% and ~25% BrdU+ cells respectively, similar to the phenotype caused by electroporation of mPtc1$^{\Delta loop2}$ alone (Fig. 2C,D; Table S1). Conversely, transfection of Wnt1/3a or the β-catenin^{CA} construct alone increased the percentage of BrdU+ cells to ~60% and ~69% respectively and resulted in overgrowth of the neural tissue (Fig. 2B,D; Table S1).

In the absence of Shh signalling, Gli proteins are proteolitically processed to generate transcriptional repressors (Jacob and Briscoe, 2003). Recently, repressor forms of Gli3 were shown to physically interact and inhibit β-catenin activity (Ulloa et al., 2007). To test whether this mechanism was operating in the developing neural tube, we electroporated a chimaeric transcriptional activator formed by the HMG box DNA-binding domain of Tcf3 fused to the VP16 transactivator of herpes simplex virus, TCF3^{VP16}, that activates transcription independent on β-catenin activity. Electroporation of TCF3^{VP16} alone resulted in increased BrdU incorporation (~53% of BrdU^{+} cells 24h-PE) and overgrowth of the NT similar to the phenotype obtained by activation of the Wnt pathway at the level of the ligand or the β-catenin (Fig. S1C, D). However, co-electroporation of either β-catenin^{CA} or the TCF3^{VP16} constructs together with a repressor form of human Gli3 (Gli3R; Cayuso et al., 2006), still resulted in reduced BrdU incorporation to ~20% and ~21% BrdU+cells respectively (Fig. S1A-B, D; Table S1). These data indicate that loss of Wnt proliferative activity is not mediated by a direct inhibition of β-catenin by repressor forms of Gli (Ulloa et al., 2007), but instead that the Wnt-mediated control of cell cycle progression requires active Shh signalling at the transcriptional level.

To test the hypothesis of a possible transcriptional interaction between these pathways, we took advantage of a deleted form of Gli3 protein that contained only the DNA binding zinc-finger-domain (Gli^{Znf}) (Cayuso et al., 2006). Although electroporation TCF3^{EnR} alone caused a dramatic cell autonomous loss on BrdU incorporation and, co-electroporation with Gli^{ZnF} was sufficient to totally rescue the size and loss of BrdU+ cells caused by TCF3^{EnR} to control levels (~42% or ~44% BrdU+ cells 24h-PE of control pCIG vector, or TCF^{EnR}+ Gli^{ZnF})

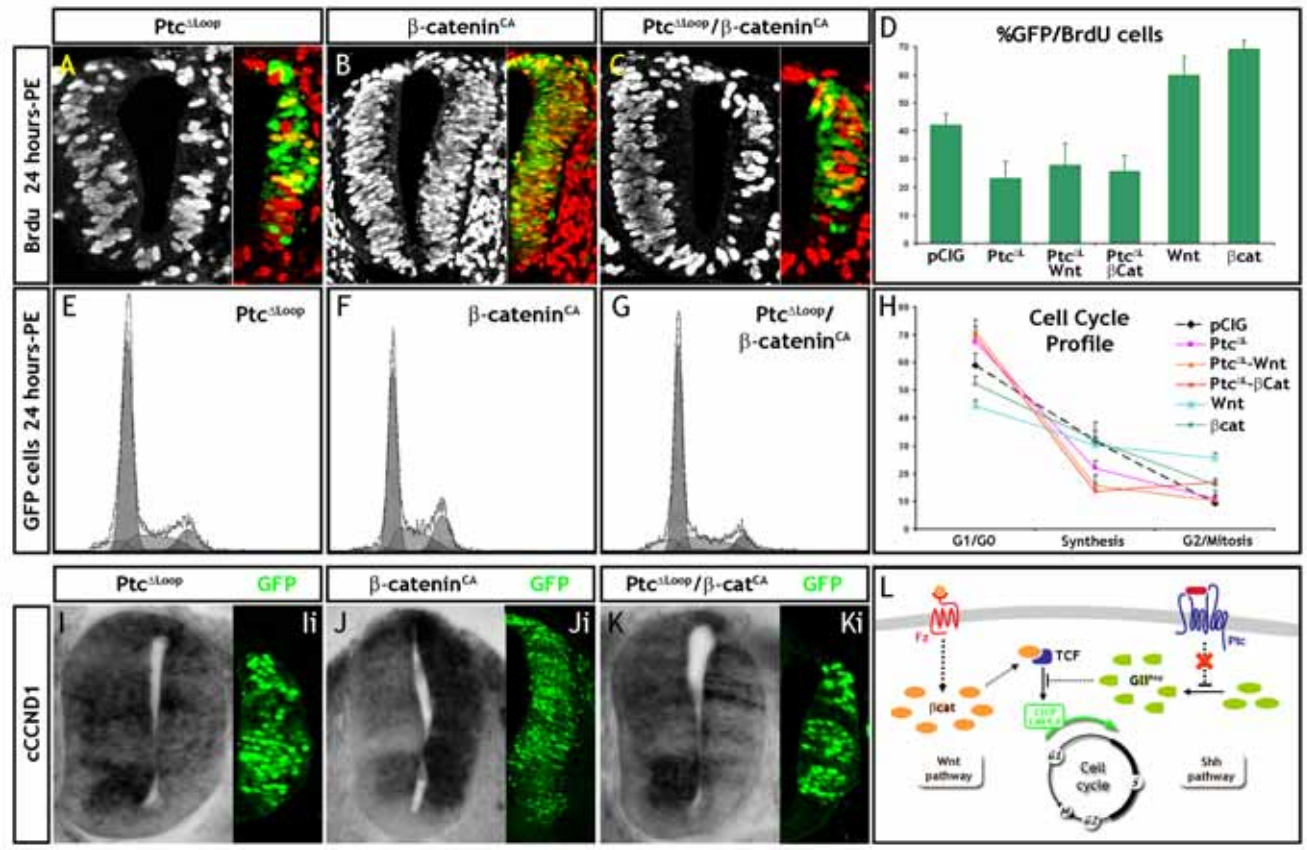

Figure 2: Shh activity is required for Wnt mediated cell cycle progression and regulation of CyclinD1 expression. (A-D) Analysis of proliferation by BrdU incorporation (red) 24h-PE of indicated DNAs (GFP, green). (A) Loss-of endogenous Shh activity by electroporation of a mutant version of the receptor Ptc, resulted in cell autonomous loss of BrdU incorporation. (B) Ectopic activation of the Wnt canonical pathway by an active version of b-catenin resulted in overgrowth of the neural tissue. (C) β-catenin losses its proliferatice capacity in a loss-of-function background for Shh. (D) Quantitative analysis of GFP-expressing cells (green) that have incorporated BrdU (red), after 30 minutes BrdU pulse, 24 hours post electroporation (h-PE) of the indicated DNAs. Analysis was carried out by separating dorsal and ventral halves of the neural tube, however since there were no significant DV differences, data are represented pooled.
(E-H) Analysis of cell cycle phase distribution of cells transfected with the indicated DNAs. (E-H) Representative examples of cell cycle profile of cells expressing mPtc$^{\Delta Loop2}$ (E), β-catenin^{CA} (F) and mPtc$^{\Delta Loop2}$+β-catenin^{CA} (G). (H) Quantitative analysis of cell cycle phase distribution after electroporation of the indicated DNAs. Loss of Shh activity resulted in increased percentage of cells arrested at the G1/G0 phase of cell cycle, as compared to control pCIG cells, however gain-of-Wnt activiy resulted in less cells in G1/G0.
(I-K) Shh and Wnt pathways regulate transcription of D-type Cyclins involved on G1 transition. (I) In situ hybridization with a chick CyclinD1 probe, 24h-PE of mPtc$^{\Delta Loop2}$. shows reduction of CyclinD1 expression. (J) 24h-PE of β-catenin^{CA} alone resulted in ectopic activation of CyclinD1 expression. (K) Co-electroporation of mPtc$^{\Delta Loop2}$+β-catenin^{CA} caused the cell autonomous reduction of CyclinD1 expression. (L) Summary of Shh/Wnt activities on the regulation of G1 progression of neuroepithelial cells.

(Fig. S2A-D). Co-electroporation of TCF^{EnR} and Gli^{ZnF} also restores control cell cycle phase distribution as assed by FACS analysis (pCIG: ~58%; Gli^{ZnF}: ~52%; TCF^{EnR}+Gli^{ZnF}: ~55% cells in G1/G0) (Fig. S2E-H). These data suggest that Wnt/TCF cell cycle control was dependent on Gli transcriptional activity.

FACS analysis of cell cycle phase distribution, showed transfected cells being arrested at the G1/G0 phase of the cell cycle, as compared to cells electroporated to the empty pCIG vector. Electroporation of mPtc1$^{\Delta loop2}$ alone or co-electroporation with Wnt1/3a or with β-catenin^{CA} increased the percentage of cells in the G1/G0 phase to ~67%, ~71%, and ~69% respectively (Fig. 2E-H, Table S1). Conversely, activation of the Wnt canonical pathway by electroporation of either Wnt1/3a, the β-catenin^{CA}, or the TCF3^{VP16} constructs alone shorten the G1 phase of cell cycle, showing only 44%, 52%, or 48% cells at G1 (Fig. 2H, Fig. S1E-H, Table S1).

Together these results show that repression of either Gli- or TCF-mediated transcription resulted in a significant proportion of cells arrested in the G1 phase of cell cycle. D-type cyclins (CyclinDs) are known to govern progression in G1, and transcriptional regulation of CyclinDs are know to depend on growth factors including Wnts (Tetsu and McCormick, 1999) and Shh (Megason and McMahon 2002; Cayuso et al, 2006).

Thus we next analysed expression of CyclinDs after activation of either the Wnt or the Shh pathways by electroporation of dominant active forms of β-catenin or Gli3 (β-catenin^{CA} and Gli3^{Act} respectively). Activation of either pathway resulted in the ectopic activation of CyclinD1 expression throughout the DV axis of the neural tube (Fig. 2J; Cayuso et al., 2006; Megason and McMahon 2002). Interestingly though, loss of Shh activity reduced expression of CyclinD1 (Fig. 2I), and in a loss-of-function background for Shh activity, β-catenin losses the capacity to activate CyclinD1 expression (Fig. 2I-K). Together these data indicate the upstream and/or combined requirement of Shh activity for proper CyclinD1 expression (Fig. 2L).

Shh activity is required for progression of G1 and G2 phases of cell cycle

Our results show that expression of CyclinD1 requires integration of Shh and Wnt activities, and that loss of either growth factor results in impaired cell cycle progression. We next sought to test whether regulation of D-type cyclins for G1 progression was the sole activity of the Wnt and the Shh pathways in growth control of the NT.

To that end epistatic experiments were done with an expression vector containing CyclinD1 (Lobjois et al., 2008) together with the constitutive transcriptional repressors forms of each signalling pathway (TCF3^{EnR} and Gli3^{R} respectively). Although electroporation TCF3^{EnR} or Gli3^{R} alone caused a dramatic cell autonomous loss on BrdU incorporation, co-electroporation with CyclinD1 was sufficient to totally rescue the loss of BrdU+ cells caused by TCF3^{EnR} to control levels (~42% or ~46% BrdU+ cells 24h-PE of control pCIG vector, or TCF^{EnR}+ CylinD1) (Fig. 3 B-D; Table S1). Interestingly however, CyclinD1 was insufficient to rescue BrdU incorporation caused by repressor forms of Gli3 (~23% BrdU+ cells 24h-PE of Gli3^{R} ; ~26% BrdU+ cells 24h-PE of Gli3^{R}+ Cyclin D1), even though ectopic expression of CyclinD1 alone resulted in a remarkably increase in proliferation to ~69% BrdU+ cells (Fig. 3A-D; Table S1). This data suggested that Gli transcriptional activity might be controlling expression and/or activity of additional factors required for cell cycle progression.

Additionally, analysis of cell cycle phase distribution, 24 hours post-transfection, showed CyclinD1 to shorten G1/G0 phase to ~41% cells in G1, while co-electroporation of CyclinD1+TCF^{EnR} resulted in cell cycle phase distribution equivalent to control pCIG electroporated cells (Fig. 3F-H; Table S2). Interestingly though co-elctroporation of CyclinD1+Gli3R resulted in ~58% cells in G1, same as in control cells, while a high ~16% proportion of cells appeared arrested in the G2/M phase of cell cycle (Fig. 3E-H; Table S2). This result suggested an additional role for the Shh pathway in the regulation of late phases of cell cycle.

To test this possibility we performed an in situ hybridization screen for additional Cyclins expressed in the developing NT under the control of either pathway. To that end either pathway were activated by the transfection of β-catenin^{CA} or with Gli3^{A}, and embryos were analysed 24 hours later for the expression of CyclinsE, CyclinsA and CyclinsB. Results showed activation of the Shh pathway to be sufficient for the over-expression of CyclinA2 (Fig. 3K), CyclinB2 (Fig. 3L), and CyclinB3 (data not shown). However, activation of the Wnt pathway, even thought caused the expected overgrowth of the NT, was insufficient for the regulation of CyclinsA/B expression (Fig. 3I,J). Levels of expression of E-type Cyclins were undetectable at this stage in the developing NT (data not shown). This analysis confirmed that the Shh pathway control cell cycle progression of neural cells by the regulation of G1 and G2 Cyclins

Altogether these data suggested that the role of the Wnt canonical pathway in the regulation of cell cycle might be restricted to the transcriptional control of CyclinD1, in a Shh dependent fashion. Sonic hedgehog instead appeared to have multiple roles in the regulation of cell cycle progression; on the one hand controlling expression of CyclinD1 or an upstream CyclinD1 regulator, on the other hand controlling the expression and/or function of G2/M regulators.

DISCUSSION

In this study different experimental strategies, including histological assessment of proliferation and

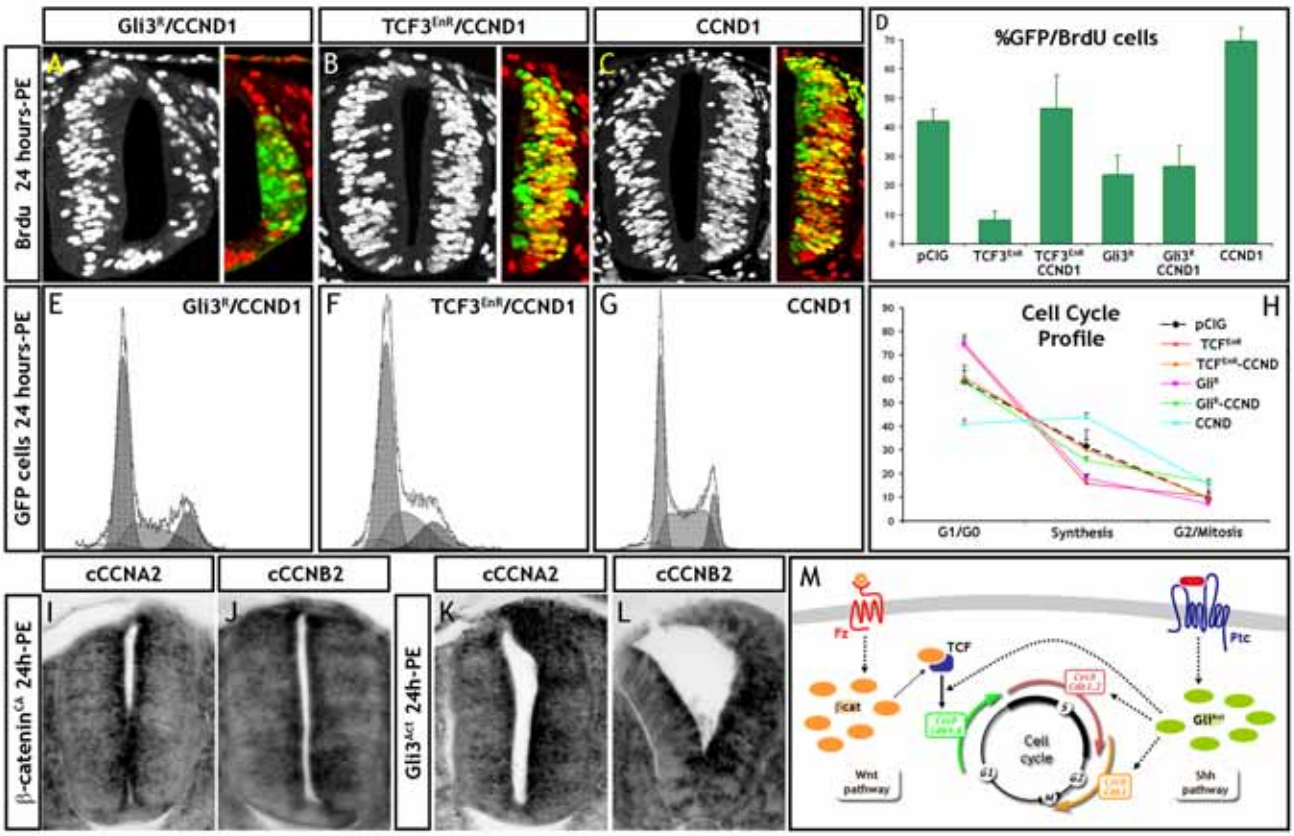

Figure 3: CyclinD1 over-expression is sufficient to rescue the Wnt-mediated cell cycle arrest but not Shh-arrest. (A-D) Analysis of proliferation by BrdU incorporation (red) 24h-PE of indicated DNAs (GFP, green). (A) Co-electroporation of CyclinD1 with a constitutive repressor of Gli transcription ($Gli3^R$) resulted in cell autonomous loss of BrdU incorporation. (B) Co-electroporation of CyclinD1 with a constitutive repressor of TCF transcription ($TCF3^{EnR}$), is sufficient to rescue normal BrdU incorporation. (C) Electroporation of CyclinD1 alone resulted in increased BrdU incorporation and overgrowth of the neural tissue. (D) Quantitative analysis of GFP-expressing cells (green) that have incorporated BrdU (red), after 30 minutes BrdU pulse, 24 hours post electroporation (h-PE) of the indicated DNAs.
(E-H) Analysis of cell cycle phase distribution of cells transfected with the indicated DNAs. (E-G) Representative examples of cell cycle profile of cells expressing $Gli3^R$ CyclinD1 (E), $TCF3^{EnR}$ + CyclinD1 (F) and CyclinD1 alone (G). (H) Quantitative analysis of cell cycle phase distribution after electroporation of the indicated DNAs. Transcriptional repression of Gli and TCF target genes resulted in increased percentage of cells arrested at the G1/G0 phase of cell cycle. Percentage of cells in each phase of the cycle is rescued to normal distribution by co-electroporation with CyclinD1 with $TCF3^{EnR}$, but not with $Gli3^R$. Instead, transfection of $Gli3^R$+CyclinD1 show a high proportion of cells at the G2/M phase.
(I-L) Expression of late A-type and B-type Cyclins is regulated by Shh but not by the Wnt pathway. (I,J) In situ hybridization with a chick CyclinA2 and CyclinB2 probes, 24h-PE of β-CateninCA showed no changes in the levels of Cyclins expression. (K,L) Electroporation of Gli3A shows increased CyclinA2 and CyclinB2 expression. (M) Summary of Shh/Wnt activities on the regulation of G1 and G2 progression of neuroepithelial cells.

flow cytometry were used with both gain- and loss-of function approaches to in vivo examine the role of Sonic hedgehog and Wnt signalling in the control of proliferation of neuroepithelial cells. Our results are consistent with a key function of Shh in the control of cell cycle kinetics, through the regulation of G1 and G2 length in neural precursor cells. Shh activity regulates the expression of key cell cycle regulators, that may account for these effects. Integration of Shh and Wnt activities is required for progression of G1, but not for the late phases of the cell cycle. Canonical Wnt activity regulates the expression of CyclinD1 in a Shh-dependent fashion, such that Shh activity is required upstream the TCF-mediated transcriptional control of CyclinD1 expression.

During neural development Shh, produced by the axial midline structures of the notochord and floor plate (Martí et al., 1995), functions as a long range signal to direct the dorsal ventral patterning of neural progenitors and control neuronal subtype identity (Jessell, 2000; Briscoe and Ericson, 2001) . In addition Shh signaling is required, in a cell autonomous manner, for growth and survival of neural progenitors (Cayuso et al., 2006). Data from our laboratory provided evidence that decreased proliferation observed on blockade of Shh signaling is restricted to those progenitors

transfected with the inhibitory constructs (mPtc1$^{\Delta loop2}$ and Gli3^{R}), while increased proliferation is restricted to cells transfected with the activating constructs (PKA^{DN} and Gli3^{Act}). This study indicated a direct influence of Shh on the control of proliferation of cells and argued against an exclusively indirect role for Shh in which Shh signaling induces the expression of another secreted molecule that acts at long range to promote the growth of the neural progenitors (Cayuso et al., 2006).

Previous studies have suggested that canonical Wnt signaling from the dorsal aspect of the neural tube via activation of β-catenin has an important role in the control of precursor cell proliferation (Megason and McMahon, 2002). The data that Shh also influences the proliferation and survival of neural progenitors raises the question of how these two mitogenic factors interact to ensure the normal growth of progenitors. It is possible that the two factors regulate the growth of the neural tube via distinct mechanisms involving different transcriptional responses. Alternatively, it is possible that both factors converge on the same set of target genes, indeed the possibility of cross talk between the two signaling should not be ruled out given the number of shared components between the two pathways (Meng et al., 2001; Jia et al., 2002; Price and Kalderon, 2002), and the number of common target genes (Megason and McMahon, 2002; Panhuysen et al., 2004). In this view cross-talk between the two pathways may act to integrate the mitogenic and survival responses of cells to ensure the well regulated growth and morphogenesis of the neural tube.

Notably, blockade of Shh signaling in both dorsal and ventral regions of the neural tube affects the survival and proliferation of progenitors (Cayuso et al., 2006). One possibility is that a Sonic, ligand independent low-level activation of the pathway in dorsal regions may be sufficient to maintain progenitor proliferation and survival. Alternatively it is possible that there is an extended range of influence of Shh that includes progenitors throughout most of the neural tube. This would be 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 neural tube (Goodrich et al., 1997). Moreover in embryos lacking Shh signaling (Chiang et al., 1996; Litingtung and Chiang, 2000; Thibert et al., 2003; Wijgerde et al 2002) the entire neural tube, not just the ventral regions appear decreased in size. Thus the long range action of Shh could be required not only for the patterning of progenitors but also for their survival and proliferation providing a means to couple these attributes of neural cells.

Our results, suggesting a wide DV requirement for Wnt signals in the control of neural precursor proliferation, together with those previously published data that indicates a similar function for Shh signalling pathway prompt us to look for possible interactions between these pathways in the growth control of the neural tube. In a series of experiments where neural precursors were blinded to see Shh, Wnt/β-catenin mediated induction of cell cycle progression is abolished in electroporated cells. Further analysis of cell cycle phase distribution by cell cytometry demonstrates that transfected cells are blocked in G1, although Wnt1/3a and dominant-active β-catenin single overexpression are able to increase proliferation rate and reduced the percentage of cell in G1 phase.

A recently published worked demonstrates that Gli3 truncated repressor forms (GLI3^{R}) can interacts with the C-terminal transactivation domain of β-catenin, and suggest that physical interaction to be responsible for a Gli3 mediated repression of the Wnt canonical pathway (Ulloa et al., 2007). In the neural tube, GLI3^{R} have been shown to strongly block cell cycle progression of neural precursors in G1 and downregulates CyclinD1 transcription (Cayuso et val., 2006). One possible explanation for our results is that the Gli3 repressor forms generated upon blocking Shh signalling could be interacting with β-catenin and thus inhibiting any possible Wnt positive signal. To overcome this question we overexpress Gli3^{R} protein together with a constitutive active form of β-catenin, that retains the Gli3 interacting domain, and with a chimaeric transcriptional activator formed by the HMG box DNA-binding domain of TCF3 fused to the VP16 transactivator of herpes simplex virus, that activates transcription downstream of β-catenin. Under these experimental conditions, neural precursors proliferation remained arrested in G1 phase, suggesting that Shh/Gli activity is repressing Wnt mediated proliferation

at the transcriptional level. This idea is consistent with opposite experiments where Wnt/TCF mediated arrest of cells in G1 is rescue by the Gli^{znf} protein co-expression. Al together these results suggest a transcriptional interaction of Wnt and Shh signalling for a coordinated control of cell cycle progression within the neural tube.

CyclinD1 is a critical component of the cell cycle machinery within the CNS (Sherr and Roberts, 2004) and represents a key link between this machinery and the extracellular signals that regulate cell cycle progression (Murray, 2004). Our results show that this Wnt/β-catenin transcriptional regulation of CyclinD1 in the neural tube context also requires the Shh signal and this result is consistent with a transcriptional interaction between Wnt and Shh signals for progressing through the G1/S checkpoint.

One possibility to explain a transcriptional interaction between Wnt and Shh pathways is that the Shh/Gli control of CyclinD1 expression in the neural tube could be mediated by another transcriptional target of Shh/Gli pathway that could be required for Wnt/β-catenin direct activation of CyclinD1 expression. One interesting candidate is N-Myc since it has been shown to control proliferation in neural progenitors (Knoepfler et al., 2002) and to responds to Shh/Gli activity in this context (Kenney et al., 2003; Cayuso et al., 2006). Myc family of transcription factors bind to E box elements in the DNA (Blackwell et al., 1990). A recently reported work has shown that an E box element located within the CyclinD1 promoter binds c-Myc and is required for linking tight junctions with cell proliferation (Huerta et al., 2007). This data opens the question of whether Shh mediated activation of N-Myc is required for Wnt transcriptional regulation of CyclinD1 promoter or not.

To analyse to what extent the Wnt- and Shh-mediated control of CyclinD1 level is sufficient to account for G1/S transition of neural precursors we overexpressed a wild type form of CyclinD1 under conditions that strongly block Wnt/TCF and Shh/Gli activity. Our results suggest that CyclinD1 overexpression is sufficient to restore normal neural progenitor proliferation profile under a Wnt lack of function background. Even though our results suggest this complete CyclinD1 rescue in BrdU incorporation and flow cytometry quantitative assays, the use of a dominant negative form of CyclinD1 suggests that G1 cyclins are not sufficient to account for the action of Wnt signalling (Megason and McMahon, 2002). However, these authors do not present this data in any way.

On the other hand, CyclinD1 overexpression was insufficient to recover neural precursor proliferation although flow cytometry analysis of cell cycle phase distribution uncovers that cell progenitors are not arrested in G1 anymore but increased the percentage of cells in G2 at the expenses of G1 and S phases. Since these cells remain in a nonproliferative state, as assessed by BrdU incorporation quantification and the reduced size of the neural tube, this result suggest an arrest in G2/M transition and an additional role of Shh in the control of this step of the cell cycle oscillator. While a role of Wnt canonical signalling in the regulation of the G2/M transition stills waiting, an Hh-mediated regulation of G2 phase of the cell cycle has been recently reported in neural precursors of the developing retina (Locker et al., 2007). Moreover, these authors showed that Hh transcriptionally activates not only cyclinD1, but also cyclinA2, cyclinB1 and cdc25C that are G2 phase activators. Additionally, Shh/Gli signalling up regulates G2/M activators cdc25b in neural tube precursors (Bénazéraf et al., 2006) and the forkhead transcription factor FoxM1 in cerebellar granule neuron precursors (Schüller et al., 2007). These published data consistently support our results and suggest a wider Hh-mediated G2/M cell cycle regulation. In addition, we first show a Shh/Gli-mediated activation of all G2/M cyclins expressed in the neural tube: cyclinA2, cyclinB2 and cyclinB3 that also support this model.

Altogether our results suggest that the current model proposing that a uniform proliferation rate along the neural tube DV axis is independently maintain by a dorsal-Wnt and ventral-Shh activities is insufficient and inaccurate. We suggest a more complex model to explain neural precursor proliferation along the DV axis that necessarily integrates Wnt and Shh activities in the control of the cell cycle machinery. In this model Wnts and Shh converge to regulate G1/S transition, while Shh/Gli pathway extends its range of action to the regulation of G2/M phase.

ACKNOWLEDGEMENTS

The authors thank Susana Usieto for invaluable research assistance. We thank Drs. for DNAs. Monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

Work in our lab is supported by the Spanish Ministry of Education, Grant BFU2007-60487/BMC and by the *Fundación "La Caixa",* Grant BM05-52-0. RA is a recipient of a FI predoctoral fellowship form Generalitat de Cataluya.

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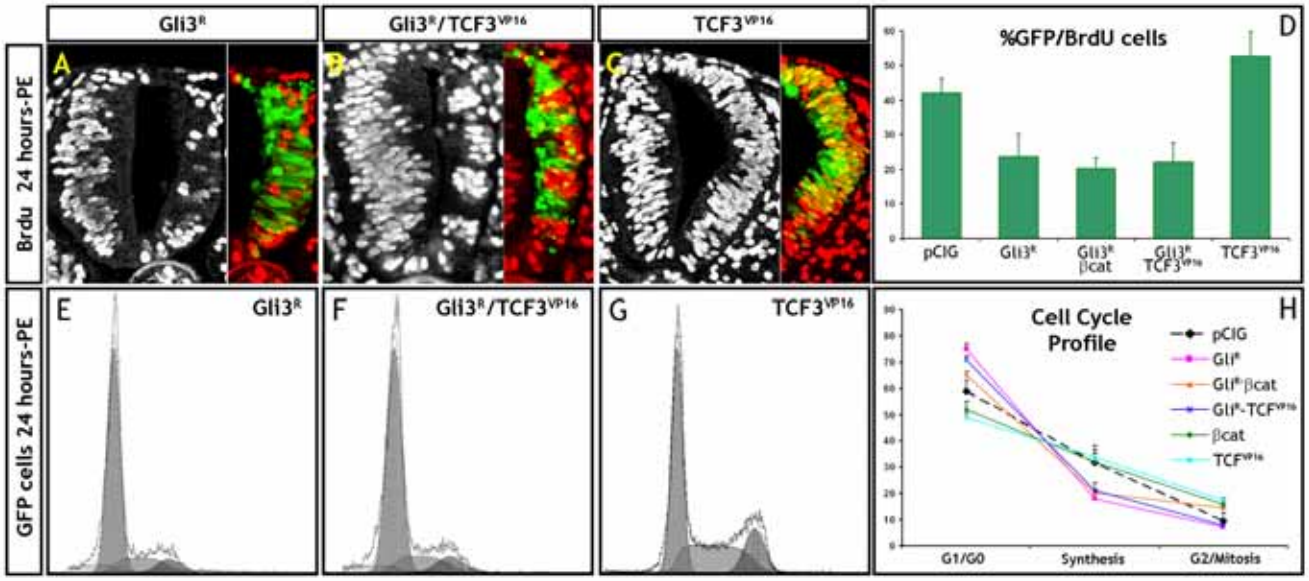
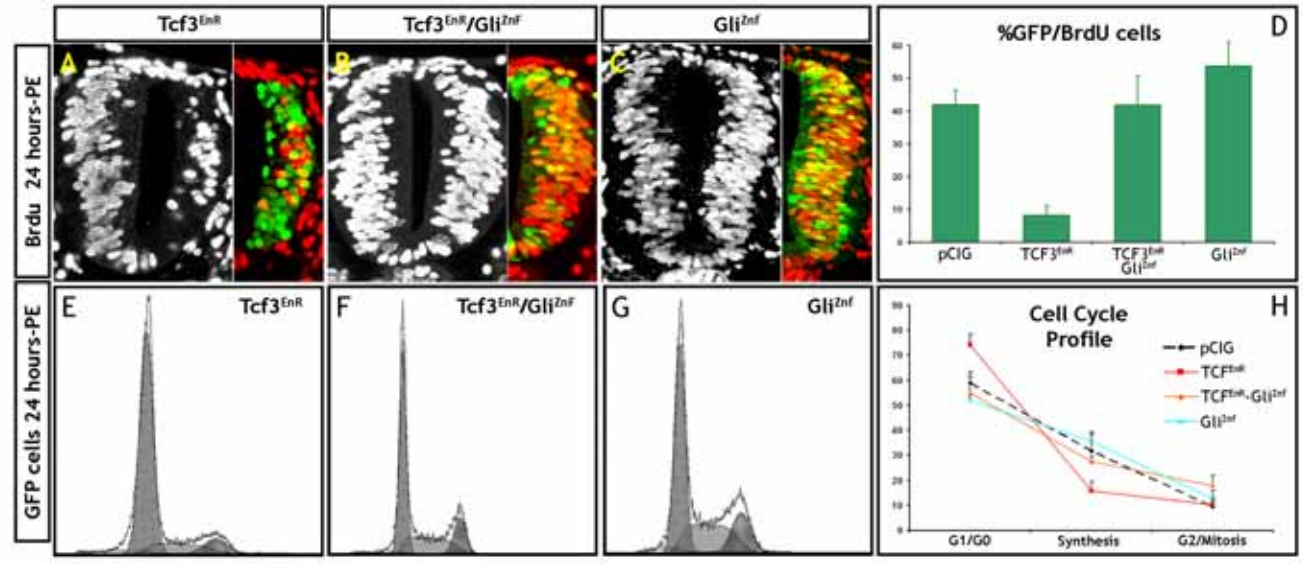
SUPPLEMENTARY FIGURES

DNA in pCIG	Statistics	Cell cycle profile 24h-PE			%GFP/BrdU 24h-PE		
		% G1/G0	% S	% G2	TOTAL	DORSAL	VENTRAL
Empty vector	Mean	58,9	31,58333	9,5	41,96700229	41,57350225	42,36050233
	s.d.	4,2993023	6,6925082	3,0403947	4,218734485	3,037388452	4,68430108
LRP6ΔC	Mean	64,34	18,8	16,74	29,76047265	30,96276447	28,55818083
	s.d.	2,7817261	4,8202697	5,8123145	8,094560112	8,133093323	8,304491349
TCF1-DN	Mean	69,6	17,225	13,15	27,67330557	30,32890695	25,0177042
	s.d.	1,9235384	2,8265114	1,1269428	3,795401533	3,262988603	2,026997667
TCF3-DN	Mean	72,5	17,9	9,6	21,00499174	21,97472573	20,03525776
	s.d.	3,6769553	1,5556349	2,1213203	6,213988199	8,571773525	3,03608392
TCF4-DN	Mean	71,26667	19,3	9,366667	18,91355199	18,23791927	19,5891847
	s.d.	3,8527047	2,9816103	0,9504385	5,974999784	5,542483361	6,415658223
TCF3-EnR	Mean	74,06	15,68	10,28	8,183704713	8,969832908	7,397576518
	s.d.	4,3517812	3,8002631	1,3736812	2,90656317	3,642818869	1,81427915
PtcΔLoop	Mean	67,36667	21,96667	10,83333	22,7884075	23,60868618	20,11957789
	s.d.	5,3463383	2,6350206	3,1214313	6,31662615	7,505641737	6,767432992
Gli3-R	Mean	74,825	17,95	7,225	23,58720625	21,10112804	24,48275862
	s.d.	2,1654484	1,4640128	1,1295279	6,697681338	5,24333256	6,592933517
PtcΔLoop/Wnt1-3a	Mean	71,06	15,68	10,28	27,73210233	28,32958165	27,13462301
	s.d.	4,3517812	3,8002631	1,3736812	7,965307303	6,756654568	9,400394486
PtcΔLoop/β-catenin-CA	Mean	69,4	13,5	17	25,52826811	25,65419057	25,40234565
	s.d.	1,9235384	2,8265114	1,1269428	5,783102599	3,599381229	7,783559964
Gli3-R/β-catenin-CA	Mean	65,13333	20,3	14,56667	20,10209461	19,21653489	20,98765432
	s.d.	1,2096832	0,781025	0,5033223	3,25809692	4,53396262	0,956292184
Gli3-R/TCF3-VP16	Mean	70,8	21,12	7,86	21,97472573	20,93629257	23,01256009
	s.d.	1,5556349	2,8917123	1,4707141	5,571773525	5,72289046	5,489976476
Gli3-R/CCND1	Mean	58,4	25,36667	16,2	26,52077948	29,90118676	28,05989
	s.d.	2,463737	1,3796135	1,3	6,990837569	3,806509101	8,11807853
TCF3-EnR/CCND1	Mean	60,36667	30,1	9,533333	46,41889594	44,04283802	48,79495387
	s.d.	5,0143128	4,1243181	5,3612809	11,26993097	16,28144128	14,84511506
TCF3-EnR/Gli-Znf	Mean	54,9	27,4	17,7	41,75195999	44,1513977	41,93788966
	s.d.	6,4346717	2,2627417	4,17193	8,869426342	12,83450298	7,77442075
Gli-Znf	Mean	51,73333	35,66667	12,56667	53,67122007	58,49764894	50,88588869
	s.d.	2,7790886	3,453018	3,5019042	7,279006229	7,008085496	7,710765844
Wnt1-3a	Mean	44,1	30,26667	25,63333	59,68000755	54,60246231	64,33442402
	s.d.	2,0663978	2,7754879	1,7009801	7,37538893	5,502486569	5,64387957
β-catenin-CA	Mean	52,06667	31,96667	15,96667	68,96988989	67,41829269	70,52148708
	s.d.	2,8589042	3,2005208	0,6806859	4,33295823	3,389119945	4,973722664
TCF3-VP16	Mean	48,83333	33,73333	17,43333	52,61076124	51,3383711	53,56505385
	s.d.	1,9091883	2,6870058	0,7778175	7,156877703	5,939462806	8,071844101
CCND1	Mean	40,93333	43,6	15,46667	69,54173987	67,85664775	71,2233729
	s.d.	1,8009257	1,9078784	0,1527525	4,576837557	4,432873884	4,720724856

Table 1. Quantitative data of percentage of neuroepithelial cells are in G0/G1 (1N DNA content), in S phase (intermediate DNA content), and in the G2/M phase (2N DNA content), 24h-PE of the indicated DNAs). Quantitative analysis of GFP-expressing cells that have incorporated BrdU, after 30 minutes BrdU pulse, 24 hours post electroporation (h-PE) of the indicated DNAs. Analysis was carried out by separating dorsal and ventral halves of the neural tube.



Supplementary figure 1: Wnt-mediated control of cell cycle progression requires active Shh signalling at the transcriptional level. (A-D) Analysis of proliferation by BrdU incorporation (red) 24h-PE of indicated DNAs (GFP, green). (A) Loss-of endogenous Shh activity by electroporation of a repressor form of Gli3 ($Gli3^R$), resulted in cell autonomous loss of BrdU incorporation. (B) Ectopic activation of the Wnt canonical pathway by the chimaeric transcriptional activator $TCF3^{VP16}$ resulted in overgrowth of the neural tissue. (C) $TCF3^{VP16}$ losses its proliferatice capacity in a loss-of-function background for Shh/Gli. (D) Quantitative analysis of GFP-expressing cells (green) that have incorporated BrdU (red), after 30 minutes BrdU pulse, 24 hours post electroporation (h-PE) of the indicated DNAs. Analysis was carried out by separating dorsal and ventral halves of the neural tube, however since there were no significant DV differences, data are represented pooled.
(E-H) Analysis of cell cycle phase distribution of cells transfected with the indicated DNAs. (E-G) Representative examples of cell cycle profile of cells expressing $Gli3^R$ (E), $Gli3^R$ +$TCF3^{VP16}$ (F) $TCF3^{VP16}$ (G). (H) Quantitative analysis of cell cycle phase distribution after electroporation of the indicated DNAs. Loss of Shh activity resulted in increased percentage of cells arrested at the G1/G0 phase of cell cycle, as compared to control pCIG cells, however gain-of-Wnt activiy resulted in less cells in G1/G0.



Supplementary figure 2: Wnt/TCF control of cell cycle is dependent on Gli transcriptional activity. (A-D) Analysis of proliferation by BrdU incorporation (red) 24h-PE of indicated DNAs (GFP, green). (A) Electroporation of a constitutive repressor of TCF transcription ($TCF3^{EnR}$) resulted in cell autonomous loss of BrdU incorporation. (B) Co-electroporation of $TCF3^{EnR}$ with a constitutive repressor of TCF transcription ($TCF3^{EnR}$), is sufficient to rescue normal BrdU incorporation. (C) Electroporation of Gli^{Znf} alone resulted in a slight increased BrdU incorporation. (D) Quantitative analysis of GFP-expressing cells (green) that have incorporated BrdU (red), after 30 minutes BrdU pulse, 24 hours post electroporation (h-PE) of the indicated DNAs.
(E-H) Analysis of cell cycle phase distribution of cells transfected with the indicated DNAs. (E-G) Representative examples of cell cycle profile of cells expressing $TCF3^{EnR}$ (E), $TCF3^{EnR}$ + Gli^{Znf} (F) and Gli^{Znf} alone (G). (H) Quantitative analysis of cell cycle phase distribution after electroporation of the indicated DNAs. Transcriptional repression of TCF target genes resulted in increased percentage of cells arrested at the G1/G0 phase of cell cycle. Percentage of cells in each phase of the cycle is rescued to normal distribution by co-electroporation with Gli^{Znf}.

DISCUSSION

Overview

Signalling pathways are an omnipresent force in every animal's life. During development, a few evolutionary conserved pathways are used time after time in different contexts, providing critical cell to cell communication and positional clues that are required to coordinate the activities of a vast numbers of cells (Pires-daSilva and Sommer, 2003). In adulthood, similar communication mechanisms are used to achieve tissue homeostasis and regeneration. Regulation of signalling is crucial; too much or too little activity from a given signal transduction pathway can cause devastating results such as developmental defects or, later in life, disease. The molecular mechanisms that integrate the different activities of these pathways in a particular time and space context is one of the main and more complex situations that needs to be profoundly understood in order to achieve the cure of most human disorders.

Wnt and Hedgehog proteins are signalling molecules that direct many aspects of metazoan development through signalling cascades that are just beginning to be understood. Interestingly enough, evidence is emerging that both pathways are similar to each other in several aspects. They are lipid-modified proteins, and some components of the signal transduction apparatus are common while others are evolutionary related (Kalderon, 2002; Nusse, 2003). These families of secreted proteins can act on neighbouring cells as short-range signals, but also as morphogens, inducing different outcomes on cells away from their source in a concentration dependent manner (Lander, 2007). In any case, there are many processes during development that are simultaneously influenced by Wnt and Hh signalling molecules.

In vertebrates, one of the systems where these two pathways converge to control cell fate and proliferative decisions is the developing spinal cord. However, an integrative model that could relate these activities with both Wnts and Shh readouts is missing. For that reason, I have focused my work in the search for possible interactions between these two important signalling pathways in the coordination of specification and proliferation of neural precursor cells.

In the neural tube, it is well known that Shh secreted from the notochord and Floor plate regulates the expression of patterning genes along the DV axis, and the combined action of this diffusible ligand and patterning factors helps to specify the types of neurons that will be born (Gomez-Skarmeta et al., 2003). Opposing activities of dorsal BMP signals and ventral Shh are required for the proper specification of the different precursor populations along the DV axis (Liem et al., 2000), however, the role that dorsal secreted Wnt ligands could exert over these early patterning genes remains largely unknown.

On the other hand, there is sufficient evidence to assume that Wnt and Shh signals are able to influence proliferative decisions in neural precursors. At the molecular level, it appears that both pathways could control the regulators of the G1/S transition of cell cycle (Megason and McMahon 2002, Cayuso et al., 2006). This raises several attractive questions; i) are these two pathways acting in parallel on different precursor populations, i.e. Wnts-dorsal and Shh-ventral? ii) are these two pathways acting within the same precursor cell, but independently, on different targets to control cell cycle progression? iii) are components of these two pathways interacting upstream of the transcriptional control of target genes? In vertebrates, at least two of such molecular interactions have been reported in different cellular contexts between Sufu and β-catenin (Meng et al., 2001) and between Gli3 repressor form and β-catenin (Ulloa et al., 2007). Moreover, in Drosophila the Hh and Wnt pathways share the role of Gsk3β (Jia et al., 2002; Price and Kalderon, 2002).

The pros and cons of the chicken model system

To search for the possible interplay between Wnt and Shh signalling during development of the neural tube we select the chicken as the principal model system. The chicken embryo has served as a classical model system in a wide range of developmental processes that includes gastrulation, somitogenesis or limb and CNS development (Stern, 2004). This vertebrate animal model has two main advantages. First, there

is an easy access to the embryo in vivo and is easy to maintain after the manipulation. Second, it is fast and easy to misexpress genes in the chicken embryo through two mayor techniques: retroviral infections and in ovo electroporation (Somia, 2004; Nakamura et al., 2000). The latter has been extensively used for the study of the neural tube development due to the inherent morphology of this tissue that allows an easy microinjection and electroporation (reviewed in Krull, 2004).

In ovo electroporation can be use in gain of function experiments, by overexpressing full-length or dominant active forms of genes, and in loss of function experiments, by transfecting dominant negative forms. Moreover, protein knockdown experiments with morpholinos and RNA interference in the chick neural tube have been recently developed (Pekarik et al., 2003; Kos et al., 2001), although some difficulties need to be solved for an extensive use of these two approaches (Krull, 2004). In addition, during this work we have improved and validated the use of chick in ovo electroporation in the neural tube for: a) in vivo measure of pathway interactions by quantitative luciferase assays, b) in vivo spatio-temporal analysis of the activity of putative conserved enhancer modules and c) for quantification of cell cycle phase distribution by flow cytometric analysis of electroporated cells.

On the other hand, the chicken model has the principal disadvantages of lacking stable transgenic lines and the evident farther evolutionary distance to humans compare to the mouse model.

The combined use of overexpression plasmids, as DNA vehicles, and in ovo electroporation give rise to a transient and mosaic transfected embryo. This have the advantage, but at the same time disadvantage, of gain or loss of function in vivo with single cell resolution in a spatio-temporal controlled way. These properties are of special application in those systems where the classical knockout mouse model has been proved insufficient to unmask the function of a particular gene. Although this problem has been partially solved by using conditional or inducible mouse models (Bockamp et al., 2002), in ovo electroporation represent a faster and easier approach to overcome this problem in the neural tube.

Another important advantage of in ovo electroporation is the possibility to co-transfect various DNA constructs at the same time with high efficiency. In our hands, in double or triple co-transfections, almost every single transfected cell incorporates the different electroporated constructs, as assessed by double or triple inmunostainnig assays (data not show). This versatile property of the system has allowed us to modulate Wnt and Hh activities at the same time, and has become crucial for some of the results obtained in this work. However, co-transfection experiments introduce many variables in the system and they are not at all times possible to control. For example, although we always use equal concentration for every transfected DNA (1mg/ml) and the same vector (pCIG), we cannot be sure that the distinct co-transfected DNA constructs are expressing their respective proteins with the same efficiency. For that reason, it would be desirable to reproduce some of the co-transfection assays by generating compound Knockout mice carrying different genes knockdown at the same time. For example, generating a double-knockout/knockin mouse $Shh^{-/-}/\beta\text{-}cat^{LoxEx3/+}$ using the Brn4-cre strain, that drives expression of cre-recombinase in the spinal cord from embryonic stage E10 onwards, would express a constitutive active form of β-catenin in the spinal cord under a Shh-lacking background (Chiang et al., 1996; Zechner et al., 2003). Although the single $\beta\text{-}cat^{LoxEx3/+}$ Knockin mouse present enlarged neural tubes (Zechner et al., 2003) resembling the $\beta\text{-}cat^{CA}$ electroporated chicken embryos, the $Shh^{-/-}/\beta\text{-}cat^{LoxEx3/+}$ mouse should show a reduced neural tube size since it would mimic the $Ptc^{\Delta Loop}/\beta\text{-}cat^{CA}$ co-transfected neural tubes, that have decreased proliferation index and a concomitant smaller electroporated side of the neural tube. An alternative experimental approach that could reduce the chicken co-electroporation complexity is the mouse in utero electroporation. This technique has been recently applied in other contexts, such as the developmental eye (Garcia-Frigola et al., 2007), and it should be perfectly suitable for neural tube electroporation. This in utero electroporation would allow us to perform single standard electroporation under a stable transgenic background and extend the range of possibilities to study signalling pathway

interactions in higher vertebrates.

The first major advance toward sequencing the chicken genome was made in March, 2003, through the production and sequencing of 64 cDNA libraries from 21 different embryonic and adult tissues (Boardman et al., 2002). This led to 339.314 EST sequences that clustered into about 10.000 genes. This work added to the chicken system the possibility of rapid access to the expression pattern or level of any gene represented in the library. In this work, the majority of probes used for in situ hybridization where obtained from this library (http://www.chick.manchester.ac.uk).

In May, 2004, the first annotated version and an initial analysis of the complete chicken genome was published in Nature (International Chicken Genome Sequencing Consortium, 2004). The chicken genome is very compact, compressed by 40% with respect to the human and mouse genomes, although only two chicken protein families are absent from the human genome, while a further 21 are absent from *Fugu*. This makes it a very valuable resource for comparative genome-wide analysis, especially the identification of conserved noncoding regions, which is greatly aided by the evolutionary position of the avian linage with respect to other vertebrates. Finally, the chicken genome sequencing also made possible for the first time to design tools such as siRNAs and morpholinos for loss of function experiments (Stern, 2005).

Wnt/β-catenin pathway regulates pattern formation the neural tube

The vertebrate spinal cord can be subdivided into dorsal and ventral domains, in which functionally distinct neurons are first specified and later settled into the outer mantle layer where they terminally differentiate. Several patterning factors expressed in different and partially overlapping domains act in concert to, under specific combinations, define the particular DV position where those neurons differentiate. Although the expression pattern along the DV axis of these progenitor markers is thought to be under the control of diffusible signals such as dorsal-BMPs and ventral-Shh, the role of Wnt family of secreted proteins in this process was simply unknown.

Even though the previously published data support that Wnt canonical pathway has little effect on the expression of patterning genes in the early neural tube development (Megason and McMahon, 2002), our data suggest that this is not the case. One possible explanation for these controversial results is that in the Megason and McMahon work, although the chicken embryos were electroporated at an equivalent stage (HH10-11), the analysis of the expression pattern of progenitor DV patterning markers after activating the Wnt canonical pathway was performed 52 hours-PE. At this stage, the electroporated side of neural tubes is extremely enlarged after Wnt activation. This seriously difficult the study of DV pattern formation, since it is not possible to compare electroporated and non-electroporated sides of the neural tube. However, they analyse the effect of the same treatment after 34 hours-PE and observed a strong repression of motorneuron marker Islet1 which support our results. Moreover, they do not look at the effect of blocking Wnt canonical pathway on any patterning gene.

Several members of the Wnt family, including *wnt1, wnt3, wnt3a, wnt4, wnt5b* and *wnt7a* or *b*, are expressed at different levels of the DV axis of the neural tube in both mouse and chick embryos (Hollyday et al., 1995; Megason and McMahon, 2002; Parr et al., 1993; Summerhurst et al., 2008). Our results suggest that Wnt1 and Wnt3a, but not Wnt4, through the canonical pathway, regulate patterning genes expression. The activity of other Wnt ligands on this process remains to be tested although Wnt3, Wnt7a and b, together with Wnt4, have been shown to lack any pro-mitotic activity and therefore, might not be affecting β-catenin intracellular levels (Megason and McMahon, 2002). However, the activity of one or more of these other Wnt homologs could explain the fact that Wnt1/Wnt3a double knockout mouse maintain *Pax3, Pax6* and *Dbx* expression unaffected (Ikeya et al., 1997).

Furthermore, our data demonstrate that changes in expression of progenitor proteins as a consequence of Wnt activation results in phenotype changes in differentiated neurons with an increase of dorsal interneurons populations at expenses of intermediate and ventral populations. This gain of function experiment agrees with a finer analysis of the Wnt1/

Wnt3a deficient mouse where the three dorsalmost subtypes of interneurons, dI1, dI2 and dI3 are reduced, while the number of cells in dI4 and dI6 domains is double and dorsally expanded. Nonetheless, no significant changes in any ventral neuronal populations were reported. Additionally, Wnt3a induces dI1 to dI3 interneurons and represses dI4 to dI6 interneurons differentiation in vitro (Muroyama et al., 2002).

TCF factors expressed throughout the DV axis account for Wnt-mediated DV patterning

TCF/LEF family of transcription factors are the downstream effectors of the Wnt/β-catenin signal transduction pathway. We showed that TCF/LEF family members are differentially expressed in the developing neural tube: TCF1 dorsal, TCF3 intermediate, TFC4 ventral and LEF-1 almost absent at HH18 embryonic stage. Although these expression patterns were partially described in the literature (Korinek et al., 1998; Schmidt et al., 2004), these works gave a general overview of these expression patterns and it was necessary to generate our own data.

As a result of blocking Wnt transcriptional activity by overexpressing dominat-negative forms of the TCF proteins we obtained a strong ventralization of neural tube progenitors. These results complement the dorsalization phenotype obtained by the over-activation of the pathway and strongly suggest that Wnt/β-catenin signalling play an important role during early DV patterning of the spinal cord. To support these results it would be clearifying to explore the possible DV patterning defects in TCF knockout mice. Nonetheless, the TCF deficient mice generated until today have not contributed to solve this question due to two main reasons. First, the severity of the phenotypes, like the TCF3$^{-/-}$ mice that have AP axis defects and do not complete neurulation (Merrill et al., 2004), or the double TCF1$^{-/-}$/TCF4$^{-/-}$ mice that also lack caudal structures and show duplicated neural tubes, among other abnormalities (Gregorieff et al., 2004). Second, as with *Wnt* genes, the genetic redundancy of the effectors of the pathway hides the real role of these transcription factors, for instance, the TCF1$^{-/-}$/LEF1$^{-/-}$ mice exhibit defects similar to Wnt3a mutants but only when both are missing (Galceran et al., 1999). Moreover, although some of these mutants have morphological neural tube defects, a detailed analysis using proper molecular markers that permit to visualize DV patterning defects still awaiting.

Our results suggest a redundant role in DV patterning for the three *TCF* genes expressed within the neural tube since equivalent results were obtained with the different dominant negative forms used. However, distinct TCF/LEF members have been shown to undertake differential functions in various developmental contexts such as mesoderm induction in xenopus or skin lineage differentiation in mice (Liu et al., 2005; Merrill et al., 2001). More recently, distinct activities of TCF1 and TCF3 have been shown to regulate DV patterning in the zebrafish neural tube (Bonner et al., 2008). One possibility is that dominant-negative forms that constitutively repress target gene expression are masking the specific function of a particular TCF. In fact these studies were TCF proteins show differential activities were performed by knockdown experiments using morpholinos in xenopus and zebrafish, and conditional knockout strategies in mice. Nonetheless, the dominant-negative forms used in this study only lack the highly conserved N-terminal β-catenin interacting domain while keeping the more divergent C-terminal domain that should be responsible for differential activities (Roose and Clevers, 1999). Additional knockdown experiments using specific siRNAs or morpholinos against TCF1, TCF3 and TCF4 in the chicken neural tube will solve this uncertainty.

Wnt patterning activity is independent of BMP signals

A concentration gradient of BMP ligand seems to be crucial for the proper pattern formation of the dorsal region of the spinal cord (Liu and Niswander, 2005). Loss of BMP signalling causes a loss of the most dorsal cell type, the Lhx2/9-positive dI1 interneuron. This was shown by inhibition of BMP signalling in the dorsal spinal cord through forced expression of a BMP antagonist, Noggin, or by a reduction in Smad4 using siRNA (Chesnutt et al., 2004). Our experiments show that Wnt activity regulates BMP expression so we

wonder if the dorsalization caused upon Wnt activation was due to a BMP-dependent response. To answer this question we took advantage of the inhibitory effect that the extracellular protein Noggin exerts over BMP ligands. Our results show that the dorsalizing effects of Wnt1/3a overexpression are independent of any BMP ligand activity. Consistent with these data, Wnt3a induces the differentiation of dI1 to dI3 interneurons even in the presence of Noggin in vitro (Muroyama et al., 2002). Nonetheless, we cannot discard a possible Wnt-BMP pathway interaction at downstream levels. Interestingly, such an interaction has been recently shown at the Gsk3β-Smad1 level (Fuentealba et al., 2007). These authors suggest that a dorso-ventral (BMP) and antero-posterior (Wnt/GSK3) patterning gradients are integrated at the level of Smad1 phosphorylations during xenopus embryonic pattern formation.

On the other hand, a BMP-mediated activation of Wnt gene expression has been previously published (Bursty-Cohen et al., 2004; Chesnutt et al., 2004). Our results together with this BMP induced activation of Wnt ligands open the question of whether BMP dorsalizing activity is dependent or independent on Wnt signalling.

Shh/Gli activity is required for Wnt1/3a dorsal character induction

The three vertebrate Gli proteins are expressed at different DV positions within the neural tube. Although biochemical direct evidence is missing, it is believed that Shh may control the DV patterning through the regulation of the ratio between repressor and activator Gli forms. Thus, the final read-out of Gli activity may regulate the expression of the different patterning genes (Jacob and Briscoe, 2003). In fact, Gli activity is sufficient to mediate the full range of Shh responses in ventral markers (Stamataki et al., 2005). Given that Gli1 is a direct target of Shh pathway, its expression depends on Gli2 and Gli3 presence (Bai et al., 2004) and Gli1 deficient mice lack any developmental defects (Park et al., 2000). For that reason it seems that the initial ventral response of cells to Shh is mediated by Gli2 (Bai et al., 2002). On the other hand, Gli3, expressed in dorsal to intermediate neural tube, has been proposed to function primarily as an inhibitor of Shh signalling. Shh mutant mice can, to a large extend, be alleviated by abrogating Gli3 function in Shh/Gli3 double-knockout mice (Litingtung & Chiang, 2000). Moreover, DV patterning defects in Gli3 mutant are rescue by a truncated allele of Gli3 that encodes only the N-terminus of the protein-equivalent to the Gli3 repressor form, suggesting that only the repressor activity of Gli3 is required in the spinal cord (Persson et al., 2002). For that reason, regulation of Gli2 and Gli3 activity is crucial for a correct Shh signal transduction.

Our results indicate that the Wnt canonical pathway caused a rapid and maintained ventral activation of Gli3, without affecting Gli2 expression. In order to study to what extent the role of Wnt pathway was dependent on Gli activity we used a deleted form of Gli3 protein that contain only the DNA binding zinc-finger-domain (Cayuso et al., 2006) and specifically block any Gli transcriptional activity, as we demonstrated by quantitative in ovo luciferase assays. Activation of the Wnt canonical pathway in the presence of the Gli^{Znf} protein results in a partial, although substantial, rescue of Wnt1/3a mediated dorsalization of the neural tube. This result was ratified for the case of constitutive active β-catenin overpression phenotype (data not shown) and for the loss of function experiments with dominat-negative TCFs.

Together these results indicate that Wnt-mediated regulation of DV pattern formation was largely, though not exclusively, dependent on the regulation of Gli3 expression. However, the analysis of the mice mutant for Gli3 shows a weaker phenotype in DV patterning than our Wnt loss of function phenotype (Persson et al., 2002). Additionally, our data overexpressing full-length Gli3 also shows a weaker downregulation of ventral markers, Olig2 and Nkx2.2, and does not promote the ventral expansion of dorsal markers such is Pax7. Toguether these data suggest additional roles for Wnt function in DV pattern, particularly in the regulation of dorsal gene expression. One possibility is that Wnt signalling 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 Gli3 or Gli2. At present, we are performing experiments to address this question, although the lack of specific antibodies against the different Gli proteins and domains is restraining our efforts.

Another possibility is that Wnt/TCF mediated activity might regulate the DV pattern formation through direct activation or repression of some of these patterning genes. Consistent with this hypothesis, a rapid repression (8 hours-PE) of ventral markers Nkx2.2 and Olig2 was observed after Wnt pathway activation while converse experiment using dominant-negative TCF constructs caused ventrally cell-autonomous activation of the same markers. Although after 8 hours of Wnt pathway activation or repression, Gli3 expression is altered these effects might not necessarily account for the indirect proposed mechanism. Furthermore, overexpression of a deleted form of TCF3 protein that contains only the DNA binding HMG-box domain and specifically blocks any TCF transcriptional activity (as we demonstrated by quantitative in ovo luciferase assays), specifically downregulates expression of the ventral markers Nkx2.2 and Olig2 24 hours-PE, without affecting dorsal genes expression (data not shown).

These results suggest an additional direct role of Wnt canonical pathway in the early ventral patterning of the neural tube. Two recently published studies support this possibility. An in silico study, that combine genome-wide comparison analysis with transcription factor binding specificity, reported that the Nkx2.2 and Olig2 loci have putative enhancer regions containing Gli and TCF regulatory sequences (Hallikas et al., 2006). Lei et al., have showed a requirement for positive synergistic Wnt and Hh activity in the control of Nkx2.2 expression (Lei et al., 2006). These data suggest that the dorsal border of Nkx2.2 expression domain is directly restricted by a TCF4 negative adjacent activity. These authors propose that this repressor activity of TCF4 is mediated by the non overlapping expression domains of the Wnt inhibitor SFRP2 and Nkx2.2. Surprisingly the authors propose this mechanism, although SFRP2$^{-/-}$ mice have a very weak dorsal expansion of Nkx2.2 domain and it is difficult to think that a diffusible protein, such is SFRP2, could define the sharp Nkx2.2-Pax6 boundary. Moreover, the authors suggest that "…widespread constitutive activation of the canonical Wnt signalling in chick neural tube progenitors results in a limited dorsal expansion of Nkx2.2 domain,...". On the contrary, our results strongly suggest that this is not the case and we propose an antagonistic interaction between Wnt and Shh pathways in the regulation of Nkx2.2 expression. The fact that Shh and Wnt signals exhibit opposing functions in partitioning the neural plate, the somites, and the inner ear along their DV axis (Robertson et al., 2004; Borycki et al., 2000; Riccomagno et al., 2005), not only supports the antagonistic hypothesis but also could extend the Wnt regulation of Gli3 expression as a wider mechanism for opposing Wnt/Hh activities. Uncovering the accurate molecular mechanism for the integration of these activities requires further experiments, although the recently reported antagonistic and direct interaction between Gli3 and β-catenin provides an attractive working model (Ulloa et al., 2007).

Wnt/β-catenin signalling directly regulates Gli3 expression in the dorsal neural tube

Our results showing that dominant-negative TCF proteins resulted in the loss of Gli3 expression (without inducing changes in Gli2 mRNA levels), together with the dose dependent expression of Gli3 in Wnt1/Wnt3a deficient mice, demonstrate that dorsal endogenous Wnt1 and Wnt3a proteins are required for Gli3 expression. Consistent with these results, an in vitro approach, using segmental plate explants, shows that a negative to positive switch of Wnt/β-catenin activity is required for Gli3 activation prior to somite formation (Borycki et al., 2000).

To explore the molecular mechanism that could account for the direct Wnt/β-catenin control of Gli3 transcription, we first search for highly conserved non-coding DNA regions (HCNR) within the human Gli3 locus that could work as potential enhancer modules, and second check whether these regions could contain closely matched core consensus TCF/LEF-binding sequences (van de Wetering et al., 1991). Our results showed a total of 13 conserved modules among widely divergent vertebrate species. Interestingly enough, although these HCNRs are distributed across

almost the entire Gli3 locus interval, the four enhancer modules containing TCF/LEF-binding sequence were found at the 5'-terminus of the locus, upstream of the start codon and in the first and second introns. This fact could reflect an evolutionary preference to keep the gene transcriptional control close to the starting point of transcription, although this hypothesis requires to be tested in a deeper extent.

Our results show that only two of the TCF/LEF-binding containing sequences are able to recapitulate dorsal to intermediate expression of Gli3 within the neural tube. Moreover, these enhancers, HCNR2 and HCNR3, are sufficient to strongly activate Luciferase expression and constitutive-active β-catenin and dominat-negative TCF respectively activates and repress enhancer-mediated luciferase activity. These data suggest that HCNR2 and HCNR3 modules are sufficient to drive expression in the dorsal neural tube, respond to Wnt/β-catenin and require TCF activity for their strong activation. One possible explanation to the fact that HCNR1, although it contains three closely located potential TCF/LEF-binding sites, was unable to drive neither GFP expression nor luciferase activation in our assays, is that could be a problem of quality rather than quantity. A recently reported study of transcription factor binding specificity shows that TCF4 factor requires a more extended sequence (Hallikas et al., 2006), 5'-(**C/G**)CTTTG(A/T)(A/T)(**C/G**)-3', for high affinity interaction with DNA. This TCF/LEF-binding sequence is present in HCNR2 and HCNR3, but not in HCNR1.

Another possible explanation to understand why the remainder conserved modules do not drive expression in the neural tube (all except HCNR2 and HCNR3; data not shown), is that these regions could function as negative enhancers to control the expression pattern of Gli3 in a complementary manner. Transcription factors with repressor activity that bind to these HCNCRs have been recently reported (Abbasi et al., 2007). Moreover, some of these transcription factors, like Pax6, Pax3, Msx1 or Nkx6.1 are expressed in the neural tube (i.e. Ericson et al., 1997). Finally, it is also plausible that these regions that do not drive expression in the neural tube could do it in other time- and space-pattern where Gli3 is expressed like the inner ear or the limb bud systems (Bok et al., 2007; Wang et al., 2000).

Even though directed mutagenesis experiments targeting the candidate TCF/LEF-binding sites are necessary for strictly demonstrate a direct role of Wnt/TCF-mediated signalling in the control of Gli3 expression, our results consistently suggest that these is the case. Additionally, the analysis of Gli3 regulatory regions reflects the endless complexity of gene transcriptional control.

Wnt canonical activity is required for proliferation throughout the DV axis of the neural tube, and regulates progression of G1 phase of the cell cycle

It is well known that Wnt ligands are key players in the growth control of various structures of the CNS, including the neural tube (Cayuso and Martí, 2005). A particularly demanding challenge for any mechanistic model of Wnt role in growth is the finding that cell divisions occur all over the neural tube, with a pattern and rate that are approximately uniform over the entire DV axis of the neural tube. This uniformity seems to be at odds with the graded distribution and activity of Wnt proteins along the DV axis that is described by Megason and McMahon (2002), which might suggest that growth should occur preferentially in the dorsal neural tube, where Wnt activity is supposed to be highest (Megason and McMahon, 2002). We believe that this most simple model is not consistent with the growing dynamics of the neural tube and needs to be revisited.

Our results suggest that the activity of the Wnt canonical pathway is homogeneous along the dorsoventral axis of the neural tube. Blocking the pathway at the receptor level and thus blinding the cells to Wnt ligands, or dominant negative TCF forms, though slowing down proliferation, do not showed significant differences along the DV axis. A recently reported work in the zebrafish neural tube use the Wnt extracellular inhibitor Dkk1 to block the pathway at the ligand level and showed that, although reducing the mitotic index, no significant DV axis differences were detected (Bonner et al., 2008). All these data suggest that Wnt signalling acts as a mitogen throughout the

entire dorso-ventral extent of the spinal cord, and not solely in dorsal progenitors.

Additionally, an in vivo TCF reporter that uses GFP as reporter gene (TOP-EGFP) (Sakai et al., 2005), showed in our hands a homogeneous expression level of GFP along the DV axis. This result is in discrepancy with the data obtained by Megason and McMahon in 2002. Using a similar experimental approach, even though the experiment was performed at a later stage where neural differentiation processes are prominent, these authors proposed a dorso-ventral gradient of Wnt mitogen activity based on different GFP expression levels (Megason and McMahon, 2002). Moreover, Bonner et al. (2008), using an equivalent reporter tool in zebrafish, also showed an increased dorsal activity of reporter gene expression; though no DV growth patterning differences were observed, as mentioned above (Bonner et al., 2008). One possible explanation for these differences is that transgenic Wnt/TCF pathway reporters containing multimerized TCF binding sites should not be assumed to give a complete or definitive readout of Wnt signalling *in vivo*. Direct comparisons reveal discrepancies among different but equivalent reporters, and some important aspects of pathway activity, including target gene de-repression will not be accurately reported by such constructs (Barolo, 2006).

In the neural tube it has been suggested that Wnt/β-catenin positively regulates progenitor proliferation by promoting G1 to S through CyclinD1 transcriptional activation (Megason and McMahon, 2002; Panhuynsen et al., 2004). We empirically confirm this hypothesis showing that cells with the Wnt pathway blocked accumulates in G1 phase of the cell cycle by in vivo flow cytometric analysis of cell cycle profile of mutant cells. These results support a CyclinD1 mediated mechanism for Wnt regulation of cell cycle progression.

Shh/Gli activity is required for the Wnt regulation of cell cycle progression and expression of CyclinD1

In Shh deficient mice cell proliferation and survival defects are observed along the entire spinal cord dorso-ventral axis (Chiang et al., 1996; Litingtung and Chiang, 2000; Wijgerde et al., 2002). Moreover, elevated expression levels of Ptc1, a Shh responsive gene, are present in a broad domain of progenitors that include the dorsal neural tube (Goodrich et al., 1997), and electroporation in the chicken neural tube of a dominant-negative form of Ptc1, that can not interact with Shh, results in a reduced proliferation rate of neural progenitors throughout the DV axis (Cayuso et al., 2006). Al together, these previous works clearly suggest that the long-range action of Shh extends along the DV axis to control proliferation.

Our results, suggesting a wide DV requirement for Wnt signals in the control of neural precursor proliferation, together with those previously published data that indicates a similar function for Shh signalling pathway prompt us to look for possible interactions between these pathways in the growth control of the neural tube. In a series of experiments where neural precursors were blinded to see Shh, Wnt/β-catenin mediated induction of cell cycle progression is abolished in electroporated cells. Further analysis of cell cycle phase distribution by cell cytometry demonstrates that transfected cells are blocked in G1, although Wnt1/3a and dominant-active β-catenin single overexpression are able to increase proliferation rate and reduced the percentage of cell in G1 phase. A recently published worked demonstrates that Gli3 truncated repressor forms ($GLI3^R$) can interacts with the C-terminal transactivation domain of β-catenin, and suggest that physical interaction to be responsible for a Gli3 mediated repression of the Wnt canonical pathway (Ulloa et al., 2007). In the neural tube, $GLI3^R$ have been shown to strongly block cell cycle progression of neural precursors in G1 and downregulates CyclinD1 transcription (Cayuso et val., 2006). One possible explanation for our results is that the Gli3 repressor forms generated upon blocking Shh signalling could be interacting with β-catenin and thus inhibiting any possible Wnt positive signal. To overcome this question we overexpress Gli3R protein together with a constitutive active form of β-catenin, that retains the Gli3 interacting domain, and with a chimaeric transcriptional activator formed by the HMG box DNA-binding domain of TCF3 fused to the VP16 transactivator of herpes simplex virus, that activates transcription downstream of β-catenin. Under these experimental conditions,

neural precursors proliferation remained arrested in G1 phase, suggesting that Shh/Gli activity is repressing Wnt mediated proliferation at the transcriptional level. This idea is consistent with opposite experiments where Wnt/TCF mediated arrest of cells in G1 is rescue by the Gli^{znf} protein co-expression. Al together these results suggest a transcriptional interaction of Wnt and Shh signalling for a coordinated control of cell cycle progression within the neural tube.

CyclinD1 is a critical component of the cell cycle machinery within the CNS (Sherr and Roberts, 2004) and represents a key link between this machinery and the extracellular signals that regulate cell cycle progression (Murray, 2004). At the transcriptional level, CyclinD1 expression has been shown to be regulated by different signalling pathways under particular conditions (Coqueret, 2002). However, a more extend and direct regulation has only been extensively demonstrated for the Wnt/β-catenin pathway (Shtutman et al., 1999; Tetsu and McCormick, 1999). In the neural tube, this Wnt-mediated transcriptional control of CyclinD1 (Megason and McMahon, 2002) is also influenced by a Shh/Gli activity (Cayuso et al., 2006).

Our results show that this Wnt/β-catenin transcriptional regulation of CyclinD1 in the neural tube context also requires the Shh signal and this result is consistent with a transcriptional interaction between Wnt and Shh signals for progressing through the G1/S checkpoint. One possibility is that both signalling effectors, Gli proteins and β-catenin/TCF complex, physically converge on the CyclinD1 promoter to modulate transcription of this gene. In fact, various consensus Gli-binding sites have been mapped upstream of the human CyclinD1 promoter (Kasper et al., 2006). In the neural tube, the published DNA region containing these Gli-binding sites cloned upstream of the luciferase reporter gene was shown to poorly respond upon activation of the Shh/Gli pathway in a vivo reporter assay (data not shown). Moreover, this region is not evolutionarily conserved among vertebrate species (data not shown). Preliminary experiments using another luciferase reporter construct that contains a 1,7 kb DNA region upstream of the human CyclinD1 start codon that includes the proximal promoter with at least one evolutionarily conserved TCF-binding site (Tetsu and McCormick, 1999), but not consensus Gli-binding sites responds to Wnt/β-catenin increase, but not to Shh/Gli activity (data not shown).

An alternative possibility to explain a transcriptional interaction between Wnt and Shh pathways is that Shh/Gli could be driving the expression of a gene required for Wnt/β-catenin direct activation of CyclinD1 promoter. One interesting candidate is N-Myc since it has been shown to control proliferation in neural progenitors (Knoepfler et al., 2002) and to responds to Shh/Gli activity in this context (Kenney et al., 2003; Cayuso et al., 2006). Myc family of transcription factors bind to E-box elements in the DNA (Blackwell et al., 1990). A recently reported work has shown that an E-box element located within the CyclinD1 promoter binds c-Myc and is required for linking tight junctions with cell proliferation (Huerta et al., 2007). This data opens the question of whether Shh mediated activation of N-Myc is required for Wnt transcriptional regulation of CyclinD1 promoter or not.

Altogether our results suggest that the current model proposing that a uniform proliferation rate along the neural tube DV axis is independently maintain by a dorsal-Wnt and ventral-Shh activities is insufficient and inaccurate. We suggest a more complex model to explain neural precursor proliferation along the DV axis that necessarily integrates Wnt and Shh activities in the control of the cell cycle machinery at least at the G1/S transition.

Shh activity is required for progression of G1 and G2 phases of cell cycle

Our results point to CyclinD1 as the principal candidate for a Wnt-Shh interaction in the control of neural precursor proliferation. To analyse to what extent the Wnt- and Shh-mediated control of CyclinD1 level is sufficient to account for G1/S transition of neural precursors we overexpressed a wild type form of CyclinD1 under conditions that strongly block Wnt/TCF and Shh/Gli activity. Our results suggest that CyclinD1 overexpression is sufficient to restore normal neural progenitor proliferation profile under a Wnt lack of function background. Even though our results suggest this complete CyclinD1 rescue in BrdU incorporation

and flow cytometry quantitative assays, the use of a dominant negative form of CyclinD1 suggests that G1 cyclins are not sufficient to account for the action of Wnt signalling (Megason and McMahon, 2002). However, these authors do not present this data in any way.

On the other hand, CyclinD1 overexpression was insufficient to recover neural precursor proliferation although flow cytometry analysis of cell cycle phase distribution uncovers that cell progenitors are not arrested in G1 anymore but increased the percentage of cells in G2 at the expenses of G1 and S phases. Since these cells remain in a nonproliferative state, as assessed by BrdU incorporation quantification and the reduced size of the neural tube, this result suggest an arrest in G2/M transition and an additional role of Shh in the control of this step of the cell cycle oscillator. While a role of Wnt canonical signalling in the regulation of the G2/M transition stills waiting, an Hh-mediated regulation of G2 phase of the cell cycle has been recently reported in neural precursors of the developing retina (Locker et al., 2007). Moreover, these authors showed that Hh transcriptionally activates not only cyclinD1, but also cyclinA2, cyclinB1 and cdc25C that are G2 phase activators. Additionally, Shh/Gli signalling up regulates G2/M activators cdc25b in neural tube precursors (Bénazéraf et al., 2006) and the forkhead transcription factor FoxM1 in cerebellar granule neuron precursors (Schüller et al., 2007). These published data consistently support our results and suggest a wider Hh-mediated G2/M cell cycle regulation. In addition, we first show a Shh/Gli-mediated activation of all G2/M cyclins expressed in the neural tube: cyclinA2, cyclinB2 and cyclinB3 that also support this model.

These results, together with the inability of Wnt/β-catenin to activate G2/M cyclins that we show and previously reported Megason and McMahon (2002), suggest a wider model for cell cycle machinery regulation in the context of the neural tube where Wnts and Shh converge to regulate G1/S transition, while Shh/Gli pathway extends its range of action to the regulation of G2/M phase.

Integrating DV pattern formation and Growth of the neural tube through Wnt and Shh opposing activities

Up to now, the knowledge of the development of practically every particular organ or tissue has been manly established by reductionist approaches based on the analysis of the role of single transduction signalling pathway. On the other hand, System Biology represents a promising and encompassing field, although it stills far from solving any empirical situation. Moving toward these two faces of the same coin, the current understanding of the biochemistry of signalling pathways together with their individual role in some well studied developmental systems, open the possibility to go into pathway interactions in depth. This approach is just to be born and may be fundamental to answer infinite questions that float in the ocean of human diseases such as cancer or degenerative disorders.

One of the vertebrate systems where this interesting approach is possible is the developing neural tube. Our work proposes two models that independently account for the integration of Wnt and Shh signalling in pattern formation and growth. However it seems much more difficult to ensemble these parallel models into a single body. An approach that could clarify the future way to follow comes from one of the existing models that try to explain how Dpp, in the Drosophila wing disc, acts as a morphogen gradient to control AP patterning, but at the same time, its graded activity maintain uniform cell proliferating in a constant rate across the anteroposterior axis (Serrano and O'Farrel, 1997). This model assumes that an inhibitor of cell proliferation has to be secreted together with the morphogen signal to form a gradient in parallel with that of the morphogen, such that all cells across the wing imaginal disc are subjected to the same growth stimulus. The expression of such an inhibitor could either be controlled by the morphogen itself or independent of it. However, no such inhibitor has been identified in the wing disc (Affolter and Basler, 2007).

Based on this model we proposed that along the dorsoventral axis of the neural tube, the role of such secreted growth inhibitor is accounted by a dorsal Gli3 repressor graded activity, while in ventral positions

TCF4 may account for an inverse and equivalent role. Wnt mitogenic signals are expressed from the dorsal roof plate and directly promote cell cycle progression but, at the same time, independently induce the expression of Gli3 at dorsal and intermediate levels. Another mitogenic gradient is formed by secreted Shh from the floor plate, inducing graded cell proliferation in the ventral neural tube and converting Gli3 expression into an intracellular parallel gradient of negative dorsal Gli3 activity. In such scenario, a graded Gli3 repressor effect might counteract and soft the Wnt mitogenic gradient activity over dorsal progenitors. At the ventral level, TCF4 is expressed in an expression pattern that is almost identical to that of Ptc1 or Gli1, which are direct targets of Hh signal. Whether TCF4 is direct target of Shh or not needs to be demonstrated but in this model, a graded negative ventral TCF4 activity caused by the converse Wnt positive gradient may directly repress cell cycle progression. In this way, Shh ventral induction of cell proliferation may be also smoothed by a parallel graded TCF4 negative contribution. In such a manner, Wnt and Shh signalling may both promote cell cycle progression, but at the same time, opposing activity among them, that are necessary to establish a correct dorsoventral patterning, serve to soft their mitotic outcomes and maintain an approximately uniform rate over the entire dorsoventral axis of the neural tube. This might of especial relevance in the more dorsal and ventral positions of the neural tube since morphogen gradients tend to decay exponentially rather than linearly (Lander, 2007).

Although a direct evidence of a Wnt gradient in the neural tube is missing, two recently published works provide new indirect and direct evidence (Dessaud et al., 2007 ; Chamberlain et al., 2008) of the presence of a Shh gradient in the ventral neural tube. Chamberlain et al., genetically engineered mice that produce bioactive, fluorescently labelled Shh from the endogenous locus. They showed the spatio-temporal formation of the gradient that at early stages is restricted to the ventral neural tube where it is necessary for early activation of ventral patterning genes and later, cover almost the dorsoventral axis in a graded fashion. This results are consistent with the necessity of integrate pattern formation and growth responses in the context of morphogen gradient outcomes.

At the light of this model, it is possible to join the distinct, but integrative activities that Wnt and Shh exert over pattern formation and growth of the neural tube. In the former, Shh expressed from the floor plate directly activate patterning genes in a concentration dependent manner while Wnt1 and Wnt3 secreted from the roof plate will activates Gli3 expression that is necessary to restrict Gli-mediated activation of patterning genes and thus sharpening the boundaries among the different neural precursor domains. In the latter, this antagonistic relation between Wnt and Shh may be used to smooth the mitogenic gradients of both pathways cross-generating opposing intracellular gradients that restrict both dorsal and ventral mitogenic activities.

Alternatively to this model, a low concentration level of Wnts or Shh could extend along the entire DV axis of the neural tube. One possibility is that a basal concentration level of these factors might be sufficient to keep neural precursors proliferating close to the saturating state, while higher concentration levels would be required for patterning processes. In fact, at early stages of development, neural precursors are very actively proliferating population. When Wnt/β-catenin or Shh pathways are constituvely activated, CyclinD1 levels increased and neural precursors are forced to divide faster. Although the resulting neural tubes are enlarged (Megason and McMahon, 2002; Cayuso et al., 2006), the proliferation rate of these cells slightly increases when compare to the strong decrease caused by the blocked of any of these pathways. This observation suggests that these precursors are right to the limit of their proliferative possibilities. Moreover, blocking Wnt and Shh pathways at the membrane level indicate that there are not significant differences along the DV axis in terms of proliferation rates, suggesting that a basal level of Wnt or Shh activities could be required along the DV axis. In this scenario, any neural precursor would require a minimal threshold of Wnt and/or Shh activity. Our results indicate that Shh signalling is fundamental to maintain any proliferative decision in these cells and that any Wnt-mediated growth control requires Shh/Gli activity.

CONCLUSIONS

1.- Dorsally expressed Wnts pattern the neural tube through the canonical signalling

2.- TCFs expressed throughout the DV axis regulate patterning of the neural tube

3.- Wnt patterning activity is independent on BMP but dependent on Shh/Gli activity

4.- Wnt signalling controls expression of Gli3 to restrict Shh/Gli activity

5.- Wnt canonical activity is required for proliferation throughout the DV axis of the neural tube and regulates progression of G1 phase of the cell cycle

6.- Shh activity is required for the Wnt regulation of cell cycle progression and expression of CyclinD1.

7.- In proliferation of neural progenitors, G2/M transition is independent on Wnt but dependent on Shh activity.

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