Development 135, 2489-2503 (2008) doi:10.1242/dev.009324

Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network

Eric Dessaud[1], Andrew P. McMahon[2,*] and James Briscoe[1,*]

Neuronal subtype specification in the vertebrate neural tube is one of the best-studied examples of embryonic pattern formation. Distinct neuronal subtypes are generated in a precise spatial order from progenitor cells according to their location along the anterior-posterior and dorsal-ventral axes. Underpinning this organization is a complex network of multiple extrinsic and intrinsic factors. This review focuses on the molecular mechanisms and general strategies at play in ventral regions of the forming spinal cord, where sonic hedgehog-based morphogen signaling is a key determinant. We discuss recent advances in our understanding of these events and highlight unresolved questions.

Introduction

In ventral regions of the presumptive spinal cord, the secreted molecule sonic hedgehog (Shh) acts as a long-range morphogen (Box 1) that directs the pattern of neurogenesis by conferring positional information to ventral neural progenitors (see Jessell, 2000; Lupo et al., 2006). Here, the Shh signaling pathway (Fig. 1) functions by regulating the expression of transcription factors, including members of the homeodomain (HD) protein and basic helix-loop-helix (bHLH) families (Fig. 2). These produce a combinatorial transcriptional code that delineates spatially distinct progenitor domains along the dorsal-ventral (DV) axis of the neural tube (Fig. 2B). Each domain generates one or more distinct neuronal subtypes, the identity of which is determined by the combination of transcription factors expressed by the progenitors. Establishment of the gene expression territories is a dynamic process that requires the progressive repression and induction of genes along the DV axis (Fig. 2C). As a consequence, the neural tube undergoes a progressive ventralization, with the sequential emergence of increasingly more ventral transcriptional codes. How then is graded Shh signaling translated into spatial- and temporal-specific profiles of gene activity in neural progenitors?

We divide this problem into three parts: (1) production and spread of Shh – the regulation of Shh distribution in the neural tube; (2) transduction of the Shh signal – the conversion of the extracellular gradient into intracellular signals that initiate cellular responses; and (3) regulation of gene expression – the molecular mechanisms that determine the transcriptional code in progenitors to control the identity of differentiating neurons.

These processes are not entirely independent as Shh-controlled feedback mechanisms modulate ligand distribution, transduction and progenitor specification within the ventral neural target field. Nevertheless, this division provides a convenient framework for the organization of this review.

[1]Developmental Neurobiology, National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, UK. [2]Department of Molecular and Cellular Biology, Harvard University, 16, Divinity Ave., Cambridge, MA 02138, USA.

*Authors for correspondence (e-mails: amcmahon@mcb.harvard.edu; james.briscoe@nimr.mrc.ac.uk)

Establishing an extracellular gradient of Shh

Shh is initially produced by the notochord, a rod-like population of mesodermal cells that acts as an organizing center for the overlying neural tissue and establishes an equivalent neural pattern on the left and right sides of the developing spinal cord (Echelard et al., 1993; Roelink et al., 1994). In amniotes, notochordal Shh induces a second center of Shh production within floor plate cells at the midline of the neural tube (Fig. 2A) (Marti et al., 1995; Roelink et al., 1995). In other vertebrates, the mechanism of floor plate induction appears to be less dependent on notochord-derived hedgehog (Hh) signaling (reviewed by Placzek and Briscoe, 2005). Several lines of evidence

Box 1. Shh as a graded morphogen

The morphogen concept dates back to the early twentieth century, but in its current formulation the theoretical work of Lewis Wolpert has been most influential (Wolpert, 1969; Wolpert, 1996). In particular, his 'French Flag' model has become the conventional view of a morphogen. In this model, an idealized morphogen signal is proposed to subdivide a tissue into domains of different gene expression that correspond to the colors of the French flag. The signal is envisaged to be a secreted substance that emanates from a localized source and spreads through the tissue to establish a gradient of activity. Cells respond to this signal by inducing different target genes at different concentrations. In this view, a morphogen has two distinguishing features. First, it acts on cells at a distance from its source (the signaling range). Second, it induces differential gene expression in a concentration-dependent manner. Both of these criteria are met by Shh signaling in the neural tube: blockade of Shh signal transduction in neural progenitors some distance from the source of Shh disrupts DV patterning (Briscoe et al., 2001; Wijgerde et al., 2002); and, in vitro, different concentrations of recombinant Shh protein induce different profiles of gene expression in neural cells (Dessaud et al., 2007; Ericson et al., 1997).

Nevertheless, the conventional view of a morphogen has been challenged in recent years (Jaeger and Reinitz, 2006; Pages and Kerridge, 2000). The French Flag model assumes that the responding cells are more or less inert, passive recipients of the graded signal; however, this assumption does not hold in the case of Shh signaling. Importantly, the response of cells to Shh signaling is fundamental to the generation of the morphogen response. First, Shh signaling regulates the expression of factors that influence its spread and stability; consequently, the target tissue plays an active role in shaping the gradient (see text). Second, the upregulation of negative regulators of the pathway by Shh signaling results in the gradual adaptation of cells to ongoing signaling (see text). The adaptation process has the effect of transforming the extracellular gradient of Shh signaling to an intracellular period of signal transduction. This transformation is essential for the regulation of differential gene expression by Shh. These findings support the view that the morphogen activity of Shh in the neural tube is, in part, an emergent property that relies on both ligand and the response of the target tissue. Experimental findings for other morphogens and tissues have also led to modifications and elaborations to the conventional morphogen concept (Jaeger and Reinitz, 2006).

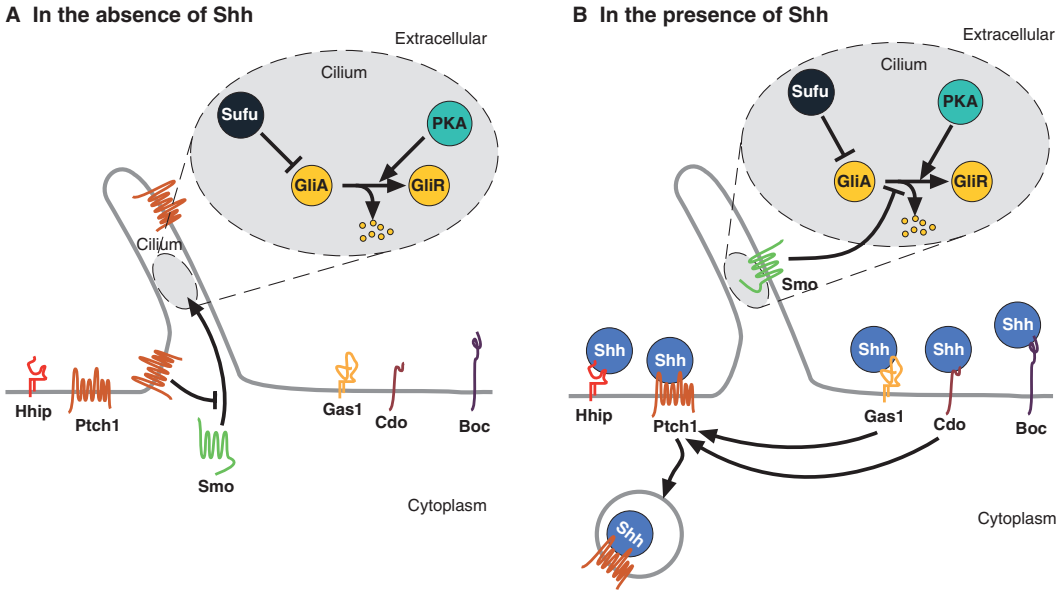

Fig. 1. Vertebrate Shh signal transduction. A summary of Shh signal transduction in vertebrates in (**A**) the absence and (**B**) the presence of Shh. (A) Patched 1 (Ptch1), a twelve-pass transmembrane protein that contains a sterol-sensing domain that binds Shh (Ingham and McMahon, 2001), represses the activity of a seven-pass transmembrane protein, smoothened (Smo), in the absence of ligand. (B) When bound by Shh, Ptch1 relieves its inhibition of Smo, allowing Smo to transduce Shh signaling intracellularly (Alcedo et al., 1996). (A) Several small sterol-like molecules inhibit or activate Smo independently of Shh. These findings, together with the similarity between Ptch1 and the RND family of bacterial transmembrane transporters, indicate that Ptch1 regulates Smo activity by moving a regulatory small molecule in or out of the cell. Cholesterol and vitamin D derivatives are possible candidates for endogenous Ptch1/Smo regulation (Bijlsma et al., 2006; Corcoran and Scott, 2006; Dwyer et al., 2007), but confirmation of this awaits further analyses. By contrast, *Drosophila* Smo is insensitive to the small-molecule modulators of vertebrate Smo (Chen et al., 2002a) and the structure of its C terminus is considerably different to vertebrate Smo, suggesting that significant differences in the mechanism of signaling have arisen during evolution (Varjosalo et al., 2006). Several additional cell surface-expressed molecules also bind to Shh, including Hhip1, which blocks pathway activation, and Cdo, Boc and Gas1, which enhance pathway activation, perhaps by increasing the presentation of Shh to Ptch1. Changes in the subcellular location of Ptch1 and Smo possibly regulate the activity of these proteins. (A) In the absence of Shh, Ptch1 localizes to cilia, and Smo is not present in cilia. (B) Upon Shh exposure, Ptch1 leaves the cilia, leading to an accumulation of Smo and to the activation of signaling. The function of cilia in hedgehog signaling is unique to vertebrates (reviewed by Huangfu and Anderson, 2006) and is essential for Shh signal transduction in the neural tube. Downstream of Smo, several proteins, including suppressor of fused (Sufu), protein kinase A (PKA) and possibly costal 2 (Cos2), are implicated in signal transduction (reviewed by Huangfu and Anderson, 2006). The exact involvement of these and other factors in vertebrates remains unclear. Although the mechanism remains to be elucidated, Smo signal transduction culminates in the regulation of Gli transcription factors, by promoting Gli activity and/or blocking the formation of Gli transcriptional repressor forms. Three Gli transcriptional regulators (Gli1, 2 and 3) are present and expressed in the neural tube, where *Gli3* expression is repressed at high Shh signaling levels (Matise and Joyner, 1999). Gli3 is a bifunctional transcriptional repressor and activator. In the absence of Shh signaling, Gli3 is proteolytically processed to generate a transcriptional repressor (GliR). Similarly, Gli2 also undergoes proteolytic processing in the absence of Shh signaling, but in contrast to Gli3, Gli2 is mostly completely degraded (yellow spots) (Pan et al., 2006). Finally, *Gli1* expression is completely dependent on Gli2/3 activator (GliA) function. Gli1 is also trafficked from the nucleus in the absence of active signaling (Sheng et al., 2006). Therefore, Shh signaling not only induces *Gli1* expression, but also regulates its nuclear accumulation and thereby its function.

indicate that the spread of Shh through the ventral neural tube of the mouse and chick embryo establishes a gradient of activity that provides crucial spatial information necessary for pattern formation. As with other morphogens, the formation of a Shh gradient depends on three processes: (1) Shh production and secretion into the target field; (2) its spread through the tissue; and (3) its degradation and removal from the tissue. Each step is tightly regulated and involves dedicated molecular machinery.

Shh lipidation affects its production and spread

The mechanism by which Shh protein is produced and released from cells is a unique feature of the Hh signaling pathway (Fig. 3). Shh undergoes a series of post-translational modifications in which the precursor protein is auto-catalytically cleaved and lipid modified (Bumcrot et al., 1995; Lee et al., 1994; Porter et al., 1995). Biologically active Shh (ShhNp) is cholesterol modified at the C terminus, palmitoylated at the N terminus and forms a high molecular weight complex (Chen et al., 2004; Pepinsky et al., 1998; Porter et al., 1996a; Porter et al., 1996b). Both lipid attachments are essential for assembly of this complex (Chen et al., 2004) and affect the activity of the secreted protein.

The release of active ShhNp from producing cells requires a multi-pass transmembrane protein, dispatched 1 (Disp1). In *Disp1*$^{-/-}$ mouse embryos, ventral neural tube patterning is severely disrupted and ShhNp is absent from the target field (Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002). How Disp1 promotes the release of ShhNp remains to be determined, but its requirement is limited to lipidated Shh; Shh lacking cholesterol (ShhN) undergoes Disp1-independent secretion (Fig. 3) (Tian et al., 2005). One possible action of Disp1 is to facilitate the assembly of soluble ShhNp multimers that are long-range, high-activity signaling complexes (Goetz et al., 2006; Zeng et al., 2001).

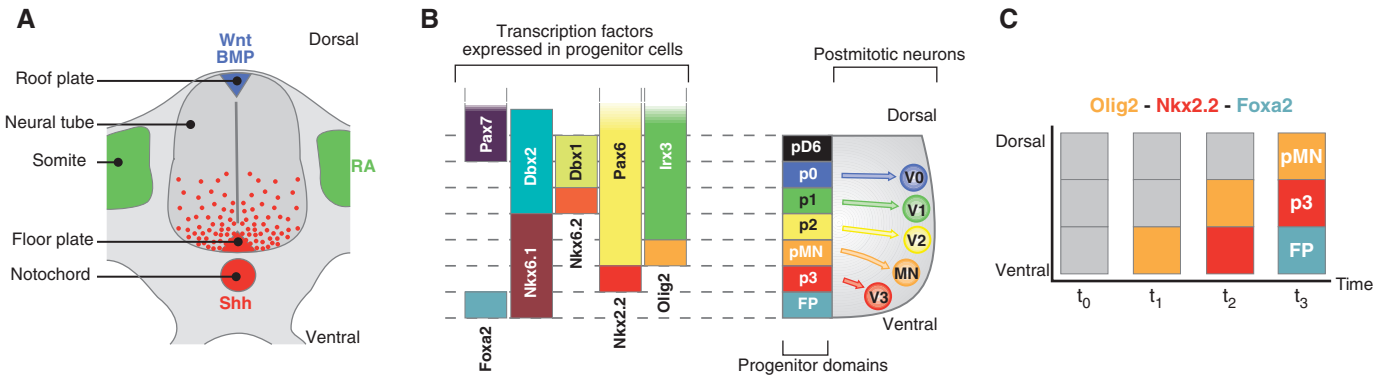

Fig. 2. Secreted signals establish the dorsal-ventral pattern of progenitor domains in the neural tube by regulating the spatial expression of transcription factors. (**A**) Schematic of a transverse section of an amniote embryo. Within the spinal cord, functionally distinct neurons are generated in a spatially segregated manner in response to signals emanating from within the neural tube and surrounding tissue. The key signals include Shh (red), secreted by the notochord and floor plate; retinoic acid (RA, green), produced by the somites that flank the neural tube; and BMP and Wnt family members (blue), which are produced dorsally. The spread of Shh from ventral to dorsal establishes a gradient of activity within the ventral neural tube (red dots). (**B**) Schematic of the ventral half of the neural tube, where the ventral gradient of Shh activity controls position identity by regulating the expression, in neural progenitors, of a set of transcription factors. These include Pax7, Pax6 and Irx3, which are repressed by Shh signaling, and Dbx1, Dbx2, Nkx6.1, Olig2, Nkx2.2 and Foxa2, which require Shh signaling for their expression. The differential response of these genes to graded Shh signaling establishes distinct dorsal and ventral boundaries of expression for each factor. The combinatorial expression of the transcription factors defines domains of progenitors (p). From the ventral pole, these are termed FP (floor plate), p3, pMN and p2-p0. Each progenitor domain is identified by its transcription factor code, and this code determines the neuronal subtype progeny the progenitors produce. Each progenitor domain generates different ventral (V) interneuron subtypes (V0-V3) or motoneurons (MN). Consequently, the spatially segregated production of distinct neuronal subtypes is determined by the DV pattern of transcription factor expression in progenitors. The ventral boundary of the progenitor domain for dorsal interneurons dI6 (pD6) illustrates the range of Shh signaling in the ventral neural tube. (**C**) The three ventral-most progenitor domains of the neural tube, FP, p3 and pMN, can be identified by the expression of the transcription factors, Foxa2, Nkx2.2 and Olig2, respectively. The onset of expression of the three transcription factors follows a dorsal-to-ventral progression, resulting in the temporally distinct establishment of each progenitor domain. Initially, ventrally located progenitors express Olig2 prior to the initiation of Nkx2.2 and Foxa2. As the expression domain of Olig2 expands dorsally, Nkx2.2 and Foxa2 are induced ventrally. Olig2 is then downregulated in cells expressing Nkx2.2. Hence, Nkx2.2 expression defines the ventral limit of the Olig2-expressing, pMN domain. Subsequently, in cells of the ventral midline, Nkx2.2 expression is downregulated by an as yet undefined mechanism. This generates a Nkx2.2$^+$ Foxa2$^-$ p3 domain, and a Foxa2$^+$ Nkx2.2$^-$ FP. One consequence of the progressive induction and modification of ventral progenitor identity is that Nkx2.2- and Olig2-expressing cells share a lineage (Dessaud et al., 2007).

The formation of the high molecular weight ShhNp complexes is likely to influence the ability of Shh to diffuse (Zeng et al., 2001). Thus, ShhN, which lacks the cholesterol moiety, would be expected to spread more rapidly and to penetrate further into the tissue. Surprisingly then, ventral neural tube patterning is compacted in mice that express ShhN in place of wild-type Shh (Huang et al., 2007; Tian et al., 2005). This might reflect a decreased range for ShhN as compared with fully processed ShhNp and/or a reduced activity of the monomeric form. Alternatively, computational modeling predicts that the high diffusion rate for an extracellular protein of the size of monomeric ShhN results in the rapid diffusion of ligand away from the site of synthesis (Saha and Schaffer, 2006). Consequently, less ShhN could be retained in the target field (as discussed below).

Palmitoylation, the second lipid modification of Shh, is catalyzed by the acyltransferase Skn (also known as Hhat). *Skn*$^{-/-}$ mouse embryos resemble *Shh* and Indian hedgehog (*Ihh*) compound mouse mutants (Chen et al., 2004; Zhang et al., 2001). Similar to ShhN, the non-palmitoylated form of Shh fails to multimerize; however, in contrast to the ShhN cholesterol-deficient signal, the non-palmitoylated form has dramatically reduced bioactivity (Chen et al., 2004; Taylor et al., 2001; Williams et al., 1999). Thus, palmitoylation might play a role beyond Shh multimerization. For example, this hydrophobic moiety might enable a high affinity interaction with Ptch1, the Shh receptor, a model consistent with hydrophobic amino acid and lipid substitutions at the N terminus of ShhN (Pepinsky et al., 1998; Taylor et al., 2001). Alternatively, the activity difference in vivo between cholesterol- and palmitoylate-deficient ShhN might reflect differential cellular release through Disp1-independent and Disp1-dependent mechanisms, respectively.

Extracellular proteins modulate Shh spread

The spread of Shh in the neural target field is influenced by the expression of extracellular and transmembrane proteins. Several classes of proteins bind to extracellular Shh protein and either restrict its diffusion or alter the rate of Shh degradation (Fig. 4). Heparan sulfate proteoglycans (HSPGs), components of the extracellular matrix, bind ShhNp at a conserved site (Rubin et al., 2002). Developmentally regulated enzymes that catalyze post-translational modifications of HSPGs (glycosylation or sulfation) might regulate ligand binding to HSPGs at different stages of development. Expression of sulfatase 1, which catalyzes the sulfation of HSPGs, correlates with the accumulation of Shh protein in the ventral lumen of the neural tube and with the dorsal expansion of Nkx2.2 expression associated with the onset of oligodendrocyte production (Danesin et al., 2006). Exploring the role of HSPGs during earlier stages of DV patterning might reveal additional functions for these proteins.

The binding of Shh to a diverse set of membrane-linked proteins has also been shown to influence the response of neural progenitors to Shh. On the basis of their effect on Shh signaling,

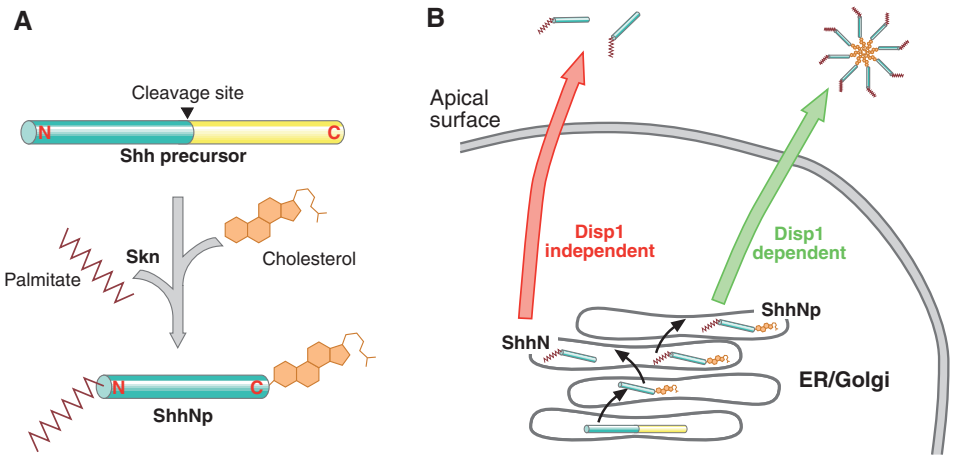

Fig. 3. Production and secretion of Shh protein. (**A**) Shh is produced as a large precursor protein that undergoes a series of post-translational modifications prior to secretion. Following cleavage of an N-terminal signal sequence upon entry into the secretory pathway, a second cleavage event, catalyzed by the C-terminal portion (yellow) of the protein, produces an N-terminal fragment (blue) that has a cholesterol adduct at its C terminus (Bumcrot et al., 1995; Lee et al., 1994; Porter et al., 1995). An acyltransferase, Skn, palmitoylates Shh on a cysteine residue near the N terminus (Chen et al., 2004; Pepinsky et al., 1998; Porter et al., 1996a; Porter et al., 1996b). These events produce a biologically active Shh, which is termed ShhNp. (**B**) The release of fully processed ShhNp from producing cells requires the multi-pass transmembrane protein dispatched1 (Disp1). How Disp1 promotes the release of ShhNp remains unknown, but it might facilitate the assembly of soluble, high molecular weight ShhNp complexes. Both palmitoylation and cholesterol modifications are essential for the assembly of this complex (Chen et al., 2004), which has long-range and high signaling activity (Goetz et al., 2006; Zeng et al., 2001). In contrast to ShhNp, an artificial construct, ShhN, that encodes the same amino acid sequence as ShhNp but lacks the cholesterol adduct is secreted from cells independently of Disp1 (Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002).

these proteins can be grouped into two classes (Fig. 4). The first class is encoded by genes that are transcriptionally upregulated by Shh signaling and includes *Ptch1*, the Shh receptor, and *Hhip1*, which encodes an EGF-repeat-containing membrane-linked protein. Their expression results in a cell-autonomous inhibition of Shh signal transduction by sequestration of ligand and, in the case of Ptch1, by endocytosis and degradation of Shh (Incardona et al., 2002). In addition, as ligand availability is decreased by sequestration, Shh signal transduction is attenuated non-cell-autonomously at distant positions in the target field (Chuang et al., 2003; Chuang and McMahon, 1999; Goodrich et al., 1997; Jeong and McMahon, 2005). Thus, a Ptch1- and Hhip1-mediated negative-feedback loop, or ligand-dependent antagonism (LDA), modulates the activity and spread of signal (Fig. 4, Fig. 5A) (Jeong and McMahon, 2005). Indeed, when LDA is inhibited, more-ventral p3 and pMN progenitors expand at the expense of more-intermediate (p0-p2) progenitors (Fig. 5A) (Jeong and McMahon, 2005). Thus, an individual cell makes a more ventral response than it should for its position in the target field. Furthermore, ectopic Shh response is observed in the dorsal region of the neural tube shortly after the initiation of Shh patterning, highlighting the importance of negative feedback in the progressive specification of a full complement of ventral cell identities. In addition, the sharpness of progenitor subtype boundaries is lost (Jeong and McMahon, 2005). Thus, upregulation of Ptch1 and Hhip1 contributes to both the spatio-temporal dynamics of patterning and the reliability of that pattern. Consistent with this, the modeling of negative-feedback mechanisms that result in enhanced ligand degradation predicts an increased robustness in the morphogen gradient (Eldar et al., 2003). Thus, LDA mediated by Ptch1- and Hhip1-dependent negative feedback might buffer fluctuations in the production and distribution of ligand and might limit the cellular response to Shh. The effectiveness of buffering might be productively explored in embryos with decreased Shh production. For example, in *Disp1* hypomorphs, a reduction in Shh protein would be predicted to reduce the dorsal expansion of ventral cell identities observed when negative feedback is attenuated, but at the same time could exacerbate the loss of precision that is associated with fluctuations in ligand distribution.

A second class of Shh-binding cell surface proteins enhance Shh signaling in a cell-autonomous manner, opposing the inhibitory activity of Ptch1 and Hhip1. These include the related transmembrane proteins Cdo (also known as Cdon) and Boc, and the glycosylphosphatidylinositol (GPI)-linked protein Gas1 (Fig. 4) (Allen et al., 2007; Martinelli and Fan, 2007; Tenzen et al., 2006; Yao et al., 2006; Zhang et al., 2006). Surprisingly, their expression is generally, but not always, inhibited by Shh signaling. Whether their cell-autonomous promotion of ventral cell fate reflects a local increase in Shh concentration or a more active role in the transduction of the Shh signaling pathway remains to be determined (Fig. 5B).

An analysis of *Cdo*, *Boc* and *Gas1* mouse mutants confirms the importance of *Cdo* and *Gas1* in the patterning of the ventral neural tube and points to the existence of functional compensation amongst these proteins (Allen et al., 2007; Martinelli and Fan, 2007; Tenzen et al., 2006). The onset of *Cdo* expression at the ventral midline of the neural tube occurs at the same time as floor plate specification (Fig. 4) and the floor plate is reduced in $Cdo^{-/-}$ mouse mutants (Tenzen et al., 2006). This suggests that Cdo might act to boost the response to Shh signaling and that this increased signal is necessary for normal floor plate induction. The deletion of *Gas1* also results in a loss of cell identities in the ventral neural tube, a defect that is enhanced in $Shh^{+/-}$ embryos (Allen et al., 2007; Martinelli and Fan, 2007). Furthermore, when *Gas1* and *Cdo* gene dosage is progressively lowered, the neural phenotype becomes progressively

more severe to the point that *Gas1*$^{-/-}$; *Cdo*$^{-/-}$ mouse embryos have no floor plate, p3 or pMN domains (Allen et al., 2007). Thus, these two proteins have cooperative, semi-redundant roles.

Together, these studies suggest a model for the role of cell surface Shh-binding proteins (Fig. 4) in which molecules, such as Cdo and Gas1, enhance Shh signaling in the target field, sensitizing cells to low levels of Shh ligand. As cells respond to Shh, these positive factors are transcriptionally inhibited while negative-feedback components are upregulated. A striking feature of this model is that the signal-dependent regulation of these proteins results in dynamic changes in the target field that influence the spread and degradation of the ligand, as well as the response of cells to the signal. The exchange enhances signaling when Shh levels are low and reduces signaling in cells exposed to high levels of ligand. This outcome might compensate for fluctuations in ligand availability, rendering a degree of robustness to the process of gradient formation. Additional molecules, such as Scube2 (Hollway et al., 2006; Kawakami et al., 2005), have been implicated in regulating the spread or activity of Shh; however, their function remains to be verified. Moreover, as no systematic effort has been made to identify all Shh binding partners, it is likely that more remain to be discovered. Both experimental testing and mathematical modeling will be necessary to gain a fuller understanding of how individual proteins shape the Shh gradient and regulate neural tube patterning.

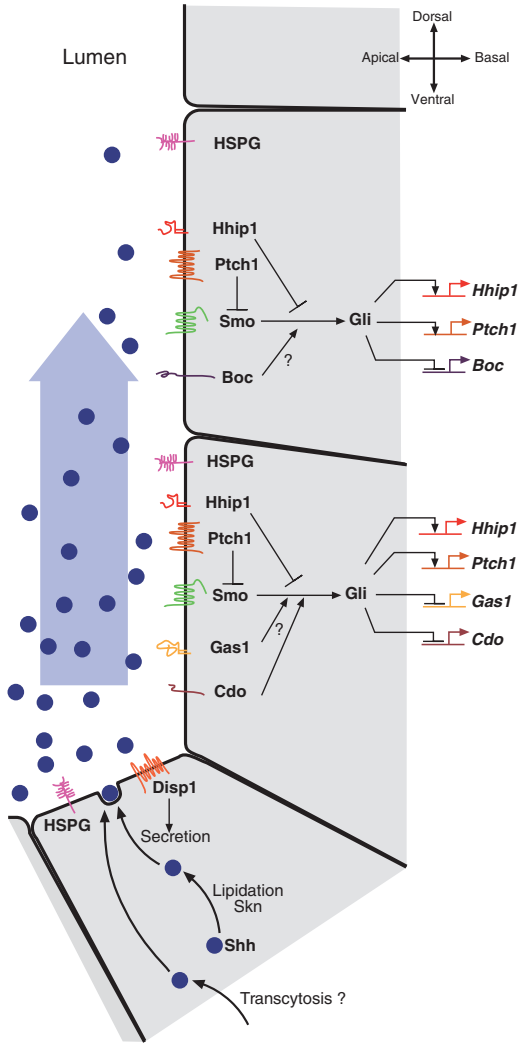

Fig. 4. Multiple proteins modulate Shh spread in the neural tube. Schematic of proteins that affect Shh spreading in the ventral neural tube. Several cell surface proteins, including HSPGs, Ptch1, Hhip1, Gas1, Cdo and Boc, interact with extracellular Shh, decreasing its spread (blue arrow) through the neural tube and either enhancing or blocking signaling in a cell-autonomous manner. HSPGs possibly bind extracellular Shh protein and slow its spread through tissue. Ptch1 and Hhip1 both bind and sequester Shh protein, and act as cell-autonomous inhibitors of signal transduction. Gas1, Cdo and Boc also bind and sequester Shh, but act cell-autonomously to promote Shh signaling. Ptch1 and Hhip1 are transcriptional targets of Shh signaling and, consequently, are upregulated in the ventral neural tube as development progresses. By contrast, the positive regulators of signaling, *Gas1*, *Cdo* and *Boc*, are transcriptionally repressed by Shh signaling. Unlike *Boc*, *Gas1* and *Cdo* are initially expressed in ventral regions of the neural tube, but are subsequently downregulated in response to Shh.

Route and timing of Shh spread

Many of the experiments described above rely on changes in the expression of Shh target genes to infer an effect on the distribution of Shh protein. This has limitations, particularly when the proteins being studied influence both the extracellular movement of Shh and the intracellular transduction of the Shh signal. It is therefore crucial to directly visualize Shh protein in the neural tube. Several studies have convincingly demonstrated the presence of Shh ligand several cell diameters from the floor plate, overlapping regions where Shh-feedback components are expressed, consistent with active, long-range signaling (Gritli-Linde et al., 2001; Huang et al., 2007). However, these studies are limited in their cellular resolution and depend on fixed tissue. The recent development of a biologically active fluorescently tagged allele of *Shh* (*Shh-GFP*), a product of the endogenous *Shh* locus, has significantly extended these initial observations (Chamberlain et al., 2008).

Shh-GFP protein generates a dynamic gradient along the DV axis of the neural tube (Fig. 5C). In *Smo*$^{-/-}$ mouse embryos, which lack a Shh response, Shh-GFP diffuses more dorsally, demonstrating the importance of negative feedback in restricting the range of Shh in the neural tube. Conversely, limited Shh-GFP was detected in *Skn*$^{-/-}$ mouse embryos, in which Shh release and/or receptor binding is diminished (Chamberlain et al., 2008). The analysis also revealed temporal changes in the level of Shh-GFP protein along the DV axis of the neural tube. Thus, cells close to the ventral midline are exposed to progressively increasing amounts of ligand, while at the same time the ligand spreads dorsally. Analyzing ligand levels in conjunction with the appearance of progressively more-ventral progenitors suggest that the duration of Shh exposure, as well as its concentration, influence the response of neural cells (see below).

Shh accumulates at the ventricular, apical pole of neural progenitors (Chamberlain et al., 2008) (Fig. 5C), an observation that raises several questions. How does a basally derived signal reach an apical position in the neural epithelium? In neural cells located in the midline of the neural tube, physically adjacent to the notochord, Shh protein is observed in small punctae closely associated with microtubules (Fig. 5C). This suggests that transcytosis might move Shh ligand received at the basal surface to an apical location (Fig. 4). The trafficking appears to be specific for Shh because a second, GFP-tagged, palmitoylated ligand (Wnt1) accumulates in basolateral positions (Chamberlain et al., 2008). Interestingly, a Disp1 homolog in *C. elegans* (CHE-14) is associated with the apical release of proteins (Michaux et al., 2000). Whether Disp1 plays a

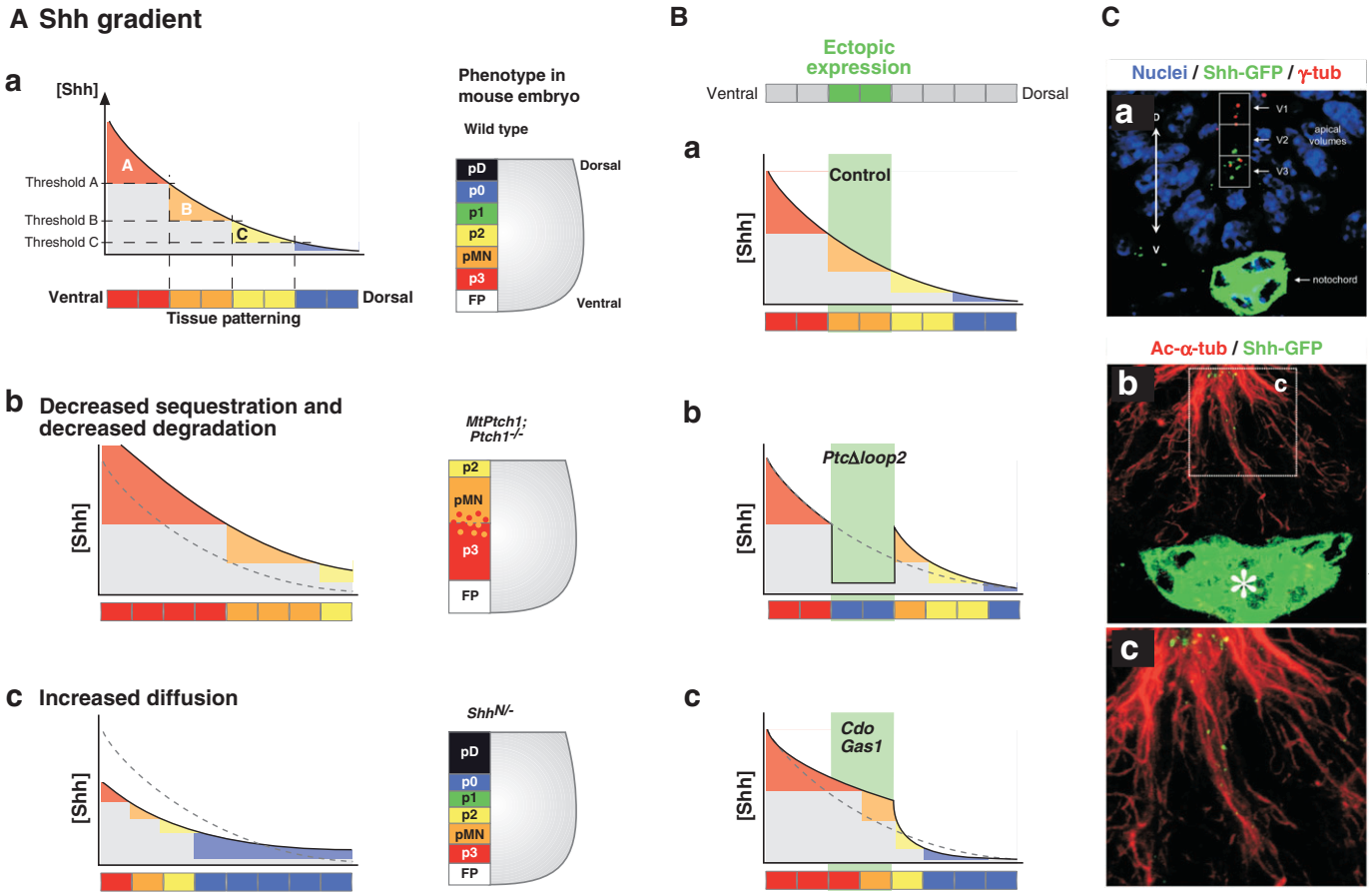

Fig. 5. Shh spread and signaling provides positional information in a graded manner to the neural tube. (**A**,a) Distinct thresholds of Shh signaling induce differential gene expression indicated by the blocks of color. Changes in the spread of Shh through the neural tube alter DV pattern. (b) Decreasing the sequestration and/or degradation of Shh by blocking the upregulation of the negative regulators Ptch1 and Hhip1 results in an increase in the amount of Shh throughout the neural tube, as compared with the control (dotted line), producing an expansion of the more ventral responses (Jeong and McMahon, 2005); see the schematic of the *MtPtch1*; *Ptch1*$^{-/-}$ mouse embryo ventral neural tube (expressing *Ptch1* under the control of the ubiquitous metallothionein promoter, *Mt*), where the floor plate (FP), p3 and pMN domains expand more dorsally than in a wild-type neural tube. (c) Increasing the diffusivity of Shh, for example by expressing the non-cholesterol-modifiable form of Shh (ShhN), results in an increased range of spread [as proposed by Saha and Schaffer (Saha and Schaffer, 2006)], compared with wild type (dotted line). A consequence of this increased range is decreased Shh accumulation close to the source of secretion and a shallower gradient, which decreases the extent of the highest responses and causes a compaction of ventral neural tube identities (Saha and Schaffer, 2006). (**B**) The forced, mosaic expression of proteins that inhibit or enhance Shh signal transduction via in ovo electroporation (ectopic expression site highlighted in green) have distinct cell-autonomous and non-autonomous effects on pattern formation in the neural tube. (a) Control electroporation (with GFP alone) has no effect on patterning. (b) The expression of Ptc1$^{\Delta loop2}$, which does not bind Shh and which inhibits signal transduction, results in a cell-autonomous blockade of signaling (compared with control, dotted line) that inhibits the generation of ventral identities. This also reduces the upregulation of Ptch1 and Hhip1, which would normally sequester Shh protein, thus increasing its spread beyond the cluster of transfected cells. (c) By contrast, the forced expression of Cdo or Gas1, which bind Shh and promote signaling, results in a cell-autonomous enhancement of responses. This increases Shh sequestration, which produces a non-autonomous reduction in the spread of Shh beyond the cluster of transfected cells, which then receive a lower amount of Shh, when compared with control electroporation (dotted line). (**C**) Shh spreads from ventral to dorsal in the neural tube to establish a gradient. (a) Within the target field, punctae of Shh protein (Shh-GFP; green) are observed apically that accumulate over time in the neural tube; first in volume v1 then in v2 and v3. Shh punctae appear to be associated with the basal bodies (marked by γ-tubulin, red) of the apically located primary cilia of neuroepithelial cells. (b,c) In neuroepithelial cells abutting the notochord (asterisk), punctae of Shh protein are observed in close proximity to microtubule fibers. The significance of this distribution remains to be determined. Adapted, with permission, from Chamberlain et al. (Chamberlain et al., 2008).

role in the release and movement of ShhNp in neural tube cells remains to be addressed. Does the apical accumulation require cells to respond to ligand? The answer appears to be no, as apical accumulation is still evident in *Smo*$^{-/-}$ embryos in which the entire target field is non-responsive. Where does ShhNp accumulate apically? Immunolocalization shows that GFP-labeled ShhNp is closely associated with the basal body, beneath the primary cilium (Fig. 5C). The association of Shh protein with cilia components might be significant given that the intracellular transduction of Shh signal requires the primary cilium (see below). Moreover, Shh basal body localization is not limited to the cells that contact the notochord, suggesting that it might represent a general mechanism

for the spread and intracellular trafficking of Shh. Alternatively, ShhNp might extend its range of action as dividing cells, which contain Shh protein, are displaced dorsally with growth of the neural tube.

The availability of the *Shh-GFP* allele also confirmed that the induction of most primary ventral neural progenitor types occurs in the absence of floor plate-derived Shh (Ding et al., 1998; Matise et al., 1999; Chamberlain et al., 2008). The role of floor plate-derived Shh might therefore be limited to later functions, such as axon guidance or glial cell-type specification (Bourikas et al., 2005; Charron et al., 2003; Danesin et al., 2006; Hochstim et al., 2008; Okada et al., 2006). Alternatively, floor plate-derived Shh might maintain DV gene expression patterns after their initial induction from a notochordal source (Danesin et al., 2006; Hochstim et al., 2008). A thorough analysis of where and for how long Shh signaling is necessary will be required.

Several additional questions remain to be addressed. Does the Shh gradient reach a steady state in which a gradient is maintained by balancing the production and degradation of Shh protein? Alternatively, does the gradient continue to evolve and, if so, is this regulated by changes in the production or spread of Shh protein? Moreover, despite the importance of ligand degradation in the establishment and maintenance of a gradient, these systems have not been thoroughly investigated. Quantitative, high-resolution, dynamic imaging of ligand production, trafficking, spread and turnover should provide new insights into these questions.

The intracellular transduction of graded Shh signaling

Once Shh has reached responding cells, how do cells perceive and respond to the signal? Despite the significant gaps in our knowledge about this signaling pathway, an understanding of how the graded signal is communicated is beginning to emerge. In particular, both the amount of Shh and the duration of signaling control pattern in the neural tube, leading to a model of how cells dynamically interpret a gradient of Shh signal.

Smoothened activation transmits the signal intracellularly

The transmembrane protein Smo molecularly links extracellular Shh ligand to the activation of its intracellular signaling pathway (see Fig. 1). Use of small-molecule antagonists and Smo agonists in neural cells indicate that a graded activation of Smo recapitulates the graded cellular response to a Shh concentration gradient (Chen et al., 2002a; Chen et al., 2002b; Dessaud et al., 2007; Frank-Kamenetsky et al., 2002). These data are compatible with the idea that increasing Shh levels progressively activate Smo. Recent studies suggest that Smo activation causes a conformational switch, relieving an intramolecular inhibitory interaction and resulting in the homodimerization or oligomerization of Smo proteins (Hooper, 2003; Jia et al., 2004; Zhao et al., 2007). In *Drosophila*, a series of mutations that increasingly weaken these inhibitory interactions progressively activate Smo (Zhao et al., 2007). Whether a similar progressive activation mechanism exists for vertebrate Smo molecules remains to be tested.

Unlike in *Drosophila*, cilia in vertebrates seem to play a key role in the intracellular transduction of Shh signal (reviewed by Eggenschwiler and Anderson, 2007). Shh signal reception removes Ptch1 from the primary cilium and the base of the cilium, thereby allowing Smo accumulation in the primary cilium (Corbit et al., 2005; Rohatgi et al., 2007) (see Fig. 1). Furthermore, suppressor of fused (Sufu) and Gli proteins are also present in cilia (Haycraft et al., 2005), and Shh ligand accumulates at the base of the cilium, perhaps in association with Ptch1 (Chamberlain et al., 2008; Corbit et al., 2005; Rohatgi et al., 2007). Whether an exchange of Ptch1 for Smo on cilia is essential for signal transduction remains to be determined. It is also unclear whether the small proportion of the total cellular pool of Smo protein present in the cilium (Rohatgi et al., 2007) is sufficient to control the entire response to Shh.

How the signal is transmitted downstream of Smo is a crucial issue that remains unresolved. In *Drosophila*, several components of the pathway have been identified (Huangfu and Anderson, 2006; Ingham and McMahon, 2001). These include a kinesin-like protein, Costal 2 (Cos2; Costa – FlyBase), which directly interacts with the cytoplasmic tail of Smo (Jia et al., 2003; Lum et al., 2003; Ruel et al., 2003). Cos2 is essential both for restraining the pathway in the absence of signal and for the activation of the Gli ortholog, Cubitus interruptus (Ci), in response to Hh. Whether the vertebrate Cos2 orthologs, Kif7 and Kif27 (Katoh and Katoh, 2004a; Katoh and Katoh, 2004b), participate in Shh signaling is contentious. In particular, there are significant sequence differences between the region of the C-terminal tail of *Drosophila* Smo that interacts with Cos2 and the equivalent region of vertebrate Smo (Varjosalo et al., 2006). Moreover, a study using short hairpin RNA knockdown in mammalian cells concluded that Cos-like function is not involved in Shh signal transduction (Varjosalo et al., 2006). By contrast, the morpholino-mediated knockdown of *kif7* in zebrafish supports a role for Kif7 in the Shh response (Tay et al., 2005). Stronger genetic models with *Kif7*-null alleles will resolve this issue. The mechanisms by which other important components of the pathway, such as Sufu, regulate signal transmission also require further investigation (Svard et al., 2006).

Graded Gli activity mediates graded Shh signaling

The signaling pathway culminates in the regulation of three members of the Gli family of zinc-finger-containing transcription regulators (reviewed by Matise and Joyner, 1999). An attractive model of Shh morphogen activity envisages graded Shh signaling evoking a gradient of Gli activity in the neural tube by progressively inhibiting Gli repressor (GliR) activity and potentiating Gli activator (GliA) function (Jacob and Briscoe, 2003). Consistent with this model, gain-of-function experiments suggest that progressive changes in the level of Gli activity are sufficient to recapitulate the patterning activity of graded Shh signaling (Lei et al., 2004; Stamataki et al., 2005). In this view, each Gli protein contributes to the production of a gradient of Gli activity that is proportional to the level of signal transduction. This implies that the sum of the activity of individual Gli proteins in the cell determines its gene expression response to Shh signaling. Therefore, the specific involvement of individual Gli proteins to the gradient depends on their intrinsic transcriptional regulatory activity, their level of expression and their post-translation regulation by Shh signaling. The changes in DV patterning in the neural tube of embryos that lack individual Gli proteins can be viewed from this perspective.

In *Gli2*$^{-/-}$ mouse embryos, the most ventral cells (the floor plate) are not specified. This suggests that Gli2 is required to generate the highest Shh signaling response, consistent with the notion that Gli2 acts predominately as an activator (Ding et al., 1998; Matise et al., 1999; Park et al., 2000). Gli3, conversely, appears to function mainly as a transcriptional repressor as mouse embryos mutant for *Gli3* exhibit a dorsal expansion of intermediate neural tube progenitors, indicating that a dorsal extension of Shh signaling has occurred (Persson et al., 2002). Moreover, all but the most ventral cell

identities (p3 and floor plate) are recovered when Gli3R function is abrogated in *Shh*- and *Smo*-null embryos (Litingtung and Chiang, 2000; Wijgerde et al., 2002). Thus, derepression via the removal of *Gli3* is crucial for the specification of MN and more-dorsal Shh-dependent progenitor domains. Interestingly, an abnormal intermixing of different progenitors is observed in both these embryos (Litingtung and Chiang, 2000; Wijgerde et al., 2002) and those lacking all Gli activity (Bai et al., 2004).

A temporal adaptation mechanism integrates the duration of Shh signaling

In addition to concentration, the duration of Shh signaling also influences DV patterning (Dessaud et al., 2007; Jeong and McMahon, 2005; Stamataki et al., 2005). A model for how both the ligand concentration and the duration of Shh signaling can control differential gene expression has been proposed (Fig. 6) (Dessaud et al., 2007). This 'temporal adaptation' model relies on a progressive decrease in the sensitivity of receiving cells to ongoing Shh signaling. Cells first appear to be highly sensitive to Shh signal. Consequently, low concentrations of Shh are sufficient to evoke high levels of Gli activity. With increasing time, cells become desensitized to ongoing Shh signaling; thus, the concentration of Shh necessary to achieve the highest levels of Gli activity increases. As a result, different concentrations of Shh generate an intracellular signal for different periods of time, such that the duration of signaling is proportional to Shh concentration. Accordingly, increasing concentrations of Shh sustain intracellular signal transduction for increasing periods of time.

The response and function of Ptch1, and perhaps other negative-feedback inhibitors, can explain the gradual desensitization of cells to ongoing Shh signaling. In response to Shh signaling, cells steadily upregulate inhibitors such as *Ptch1* (Goodrich et al., 1996; Marigo and Tabin, 1996). This suggests that increasing concentrations of Shh are necessary to block the inhibitory activity of accumulating Ptch1 (Dessaud et al., 2007). As discussed earlier, negative feedback is likely to have both cell-autonomous and non-autonomous roles in regulating the spread and response of cells to Shh (Figs 4-6). Distinguishing the relative contribution that cell-autonomous and non-autonomous processes make to normal patterning is difficult and highlights the importance of understanding the dynamics of signal transmission through the pathway. Methods that provide an ongoing measure of the activity of key components of the pathway will be essential, as will approaches for manipulating the duration of the activity of these components. Understanding how the levels and kinetics of Gli activity are altered in mutants deficient in individual Gli proteins will also be important. A key issue is to assess how the duration of signaling influences the establishment of normal DV patterning. As discussed earlier, analyses using Shh-GFP protein suggest a close correlation exists between gene induction in neural cells and the duration of their exposure to Shh (Chamberlain et al., 2008).

Dissecting the genetic network regulated by graded Shh signaling

The positional information supplied by Shh signaling regulates the spatial expression of a set of transcription factors in ventral progenitor cells (Fig. 2). These comprise a genetic network, which we will term the neural tube gene regulatory network (GRN). In common with other GRNs, there are three key issues to address. First, the members of the GRN must be identified and the molecular mechanism of their regulation uncovered. Second, we must understand how the structure and topology of the regulatory network generates the profile of gene expression observed in the neural tube. Third, the downstream targets of the network that ultimately control cell behavior and the identity of neural subtypes need to be determined. Initial progress towards these goals has been made by conventional developmental biological approaches. These have revealed specific details of the network and have suggested certain general features of its operation. The recent deployment of newer genomic and bioinformatic techniques is expanding our detailed knowledge of the neural tube GRN and is providing a broader picture of how the network functions.

Regulation of neural tube GRN genes

The initial studies that identified the HD and bHLH transcription factors that are expressed in restricted DV domains of progenitors, subdivided these regulatory factors into two classes based on their mode of regulation by Shh: class I genes are repressed by Shh, whereas class II genes require Shh exposure for their expression (Briscoe and Ericson, 2001). Many, although not all, of these factors function as transcriptional repressors (Muhr et al., 2001), and selective repressive interactions between pairs of class I and class II proteins have been defined (Figs 2, 7). As a consequence, pairs of neural tube GRN proteins delineate boundaries of progenitor domains (Fig. 7A). For example, Pax6 and Nkx2.2 comprise a cross-repressive pair that delineates the p3-pMN boundary. In mouse embryos mutant for *Pax6*, Nkx2.2 expands dorsally (Ericson et al., 1997). Conversely, Nkx2.2 expression is sufficient to inhibit *Pax6* expression, although Nkx2.2 might share this role in vivo with Nkx2.9 (Briscoe et al., 1999; Ericson et al., 1997). Similar relationships are observed between other pairs of class I and class II proteins in the neural tube. Moreover, regulatory interactions between pairs of class II proteins (Fig. 7A) have also been identified (Novitch et al., 2001; Vallstedt et al., 2001). Together, these findings reveal that an elaborate network of cross-regulation exists between members of the neural tube GRN, although how the specificity of these interactions is determined remains to be defined.

More recently, an in silico approach by Bailey et al. (Bailey et al., 2006) has extended these ideas (Fig. 7B). An analysis of evolutionarily conserved regions (ECRs) of non-coding DNA from vertebrate genomes, presumed to encode regulatory elements, found several hundred ECRs that were enriched in binding sites for HD, Sox and POU transcription factors (Bailey et al., 2006). Remarkably, many of these ECRs are close to genes that are expressed in the developing CNS, with several adjacent to established members of the neural tube GRN. This led the authors to propose that the HD transcription factor members of the neural tube GRN provide specific, direct inhibitory activity through the ECRs (Fig. 7B). Concomitantly, Sox and POU factors, which are broadly expressed in neural progenitors, provide a positive transcriptional input to confer neural expression on the genes associated with the ECRs. The distinct spatial patterns of neural tube GRN gene expression would therefore be achieved by a spatially uniform, positive transcriptional input combined with temporally and spatially restricted specific negative inputs. This mechanism is in agreement with a genetic analysis of mice mutant for certain HD transcription factors (Fig. 7C). What remains to be determined is how the DV positional information that regulates the spatial pattern of gene expression in the neural tube is incorporated into this model. The presence of other binding sites within these ECRs will doubtless be one determinant. The existence of Gli binding sites (GBSs) would presumably be important for explaining the action of graded Shh signaling (see below).

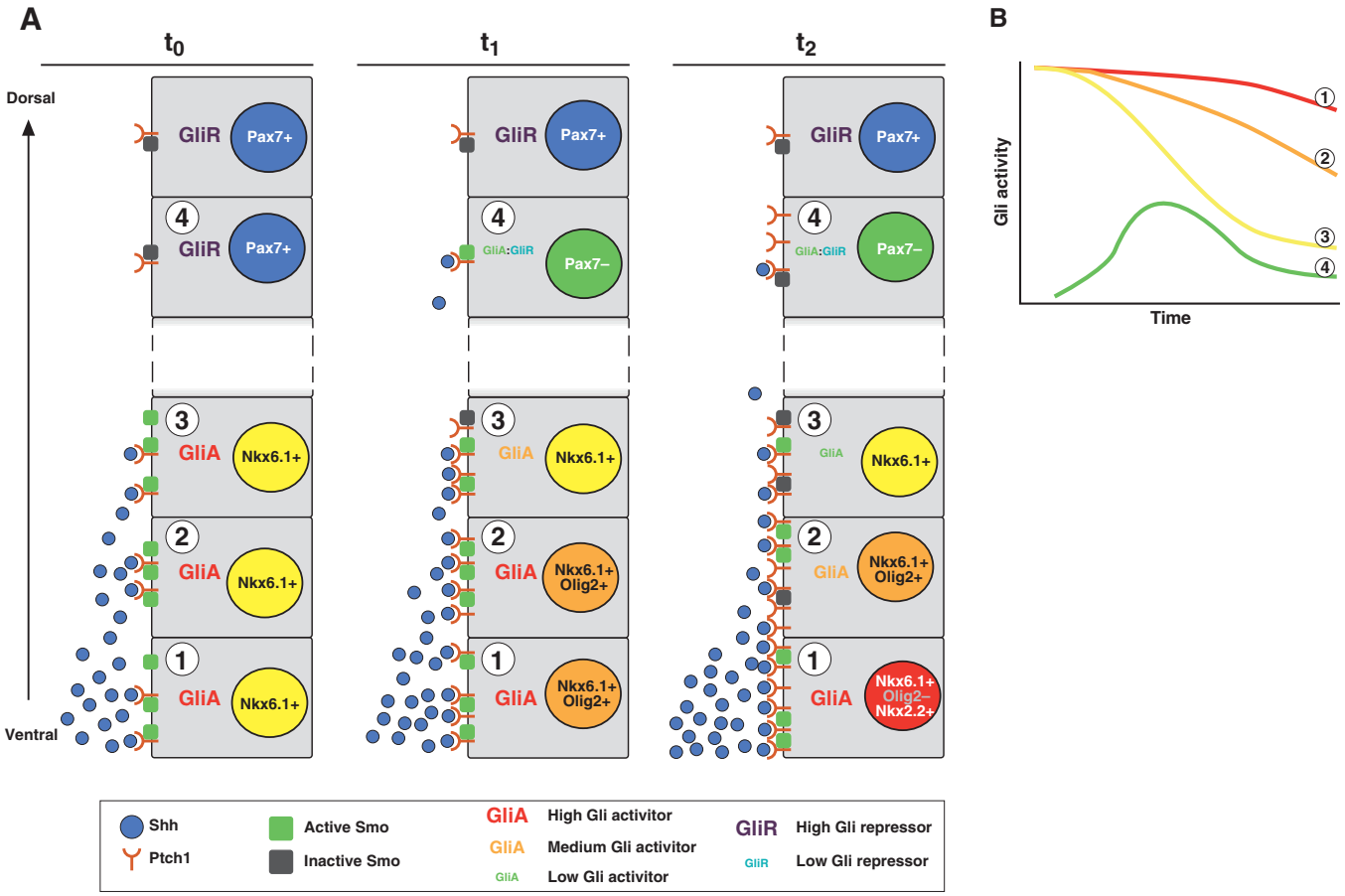

Fig. 6. A 'temporal adaptation' model for interpreting graded Shh signaling. (**A**) A model for the spatial and temporal specification of progenitor cells during exposure to Shh secreted from a ventral source. At early time points (t_0), Ptch1 expression levels (brown receptor) in neural progenitors are low, consequently low levels of Shh protein (blue) are sufficient to bind the available Ptch1. This produces high levels of Smo signal transduction (green) and, consequently, high levels of positive Gli activity (GliA, red), even in cells that are exposed to a low concentration of Shh (cell 3, t_0). The upregulation of *Ptch1* (and possibly other negative regulators of the pathway) by Shh signaling (t_1) increases the level of Ptch1 in responding cells. As a result, the concentration of Shh necessary to sustain high levels of signal transduction increases with time (t_1). In cells exposed to concentrations of Shh insufficient to bind all of the raised level of Ptch1 (cell 3, t_1), the level of GliA declines (GliA, orange). This process of cell-autonomous desensitization continues (t_2), resulting in distinct temporal profiles of Gli activity in cells arrayed along the DV axis. In addition, the upregulation of ligand-binding inhibitors of Shh signaling, including Ptch1, results in the sequestration of Shh protein in more-ventral regions of the neural tube (cell 1). Both the level and the duration of Shh-Gli activity influence the gene expression profile in responding cells. Low levels of Gli activity, for example produced by the partial inhibition of the generation of GliR activity, are sufficient to repress Pax7 (cell 4, t_1). The duration of Shh signaling is partially responsible for the distinction between Olig2 and Nkx2.2 induction. High levels of Gli activity induce Olig2 expression (cells 1-2, t_1). If the levels of signaling are sustained (cell 1, t_2), Nkx2.2 is induced and Olig2 is repressed. By contrast, if the levels of signaling in a cell decline prior to this time point, Olig2 expression is consolidated (cell 2, t_2). (**B**) The response of the indicated cells in A to Shh can be represented as a function of both Gli activity and the duration of Shh exposure (time). The adaptation of cells to ongoing Shh signaling results in different concentrations of Shh producing distinct profiles of Gli activity. Hence, temporal adaptation transforms different concentrations of morphogen into corresponding durations of increased Gli activity. In this view, the induction of each progenitor state requires exposure to a concentration of Shh above a defined threshold for a distinct period of time.

The repressive activities of neural tube GRN proteins might partly explain the temporal profiles of neural tube GRN genes. Members of the network display dynamic changes in their patterns of expression during neural tube development (Fig. 2C, Fig. 6). For individual genes, this parallels their dependence on different levels and durations of Shh signaling – that is, class I proteins are initially expressed more ventrally in the neural tube but are progressively repressed in a ventral-to-dorsal manner. The order in which class I genes are repressed is inversely related to their sensitivity to Shh signaling (Dessaud et al., 2007; Ericson et al., 1997). Thus, Shh signaling progressively defines the ventral expression boundaries of class I proteins. Class II proteins, conversely, are sequentially induced in the ventral neural tube in an order that corresponds to their increasing requirement for Shh signaling (Dessaud et al., 2007; Jeong and McMahon, 2005; Stamataki et al., 2005). These temporal features of the neural tube GRN are consistent with the importance of the duration of Shh signaling in ventral neural tube patterning. Together, the data suggest that the spatio-temporal pattern of expression for an individual neural tube GRN gene is determined by a combination of uniform positive inputs (e.g. Sox and/or POU

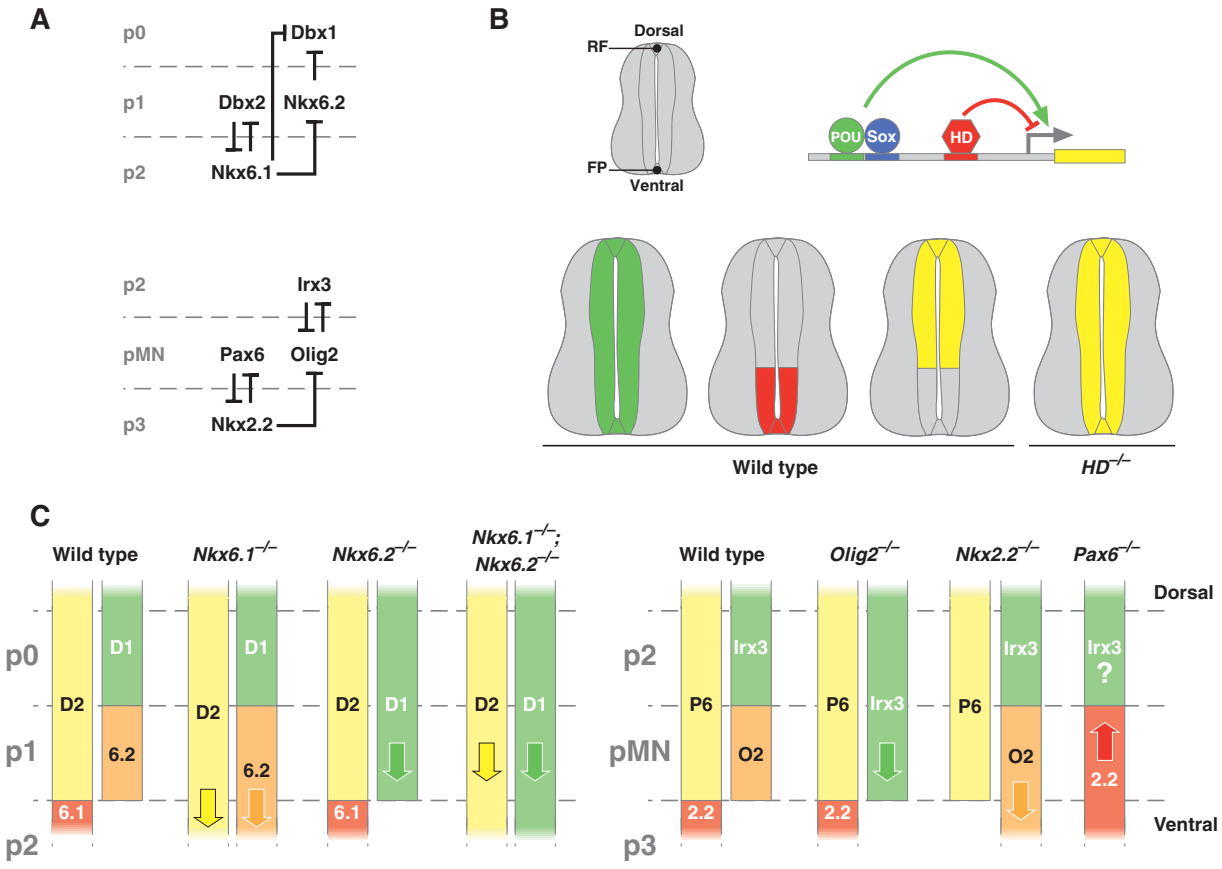

Fig. 7. The neural tube gene regulatory network. (**A**) Selective cross-repressive interactions contribute to the spatial-temporal regulation of gene expression in the neural tube. (Upper panel) Cross-repression between Nkx6 and Dbx proteins generate distinct gene expression patterns in p0, p1 and p2 domains of progenitors. (Lower panel) The generation of p2, pMN and p3 domains depends on selective cross-repressive interactions between Pax6 and Nkx2.2, and Olig2 and Irx3, and on the repression of Olig2 by Nkx2.2. (**B**) Schematic of DV patterning through repressive interactions. Representative transverse sections through mouse spinal cord are shown (RF, roof plate; FP, floor plate). The identification of regulatory modules that contain binding sites for POU, Sox and HD transcription factors adjacent to many genes of the neural tube GRN has led to a model for the regulation of these genes. POU (green) and Sox (blue) factors are transcriptional activators that are broadly expressed in neural progenitors (bottom panel, green), thus they are believed to provide spatially unrestricted activation to the regulatory modules. The HD factors (red), which have spatially and temporally restricted expression patterns in neural progenitors (example in bottom panel), mostly function as transcriptional repressors and have therefore been proposed to provide repressive input to the modules. The combination of uniform positive input and spatially restricted negative input on a target gene (yellow) would generate a spatially restricted pattern of expression (bottom panel). In this model, the consequences of mutating the repressive HD gene would be the derepression of the target gene (bottom panel, right). (**C**) Alterations in the pattern of gene expression in embryos lacking individual members of the neural tube GRN confirm the importance of cross-repressive interactions for DV pattern formation. The changes in gene expression in mouse embryos mutant for the indicated genes are summarized in the diagrams. In *Nkx6.1*$^{-/-}$ embryos, Nkx6.2 (6.2) and Dbx2 (D2) are expressed in more-ventral progenitors. Dbx1 (D1) expands ventrally in *Nkx6.2*$^{-/-}$ mutants. Deletion of both Nkx6.1 and Nkx6.2 results in the ventral expansion of both Dbx1 and Dbx2. In *Olig2*$^{-/-}$ mouse embryos, Irx3 expands into the domain normally occupied by MN progenitors. Olig2 (O2) expands ventrally in *Nkx2.2*$^{-/-}$ embryos. In embryos mutant for *Pax6*, Nkx2.2 (2.2) expands dorsally, repressing Olig2 expression (Irx3 expression has not been determined).

proteins) and spatio-temporally restricted inputs from Shh signaling, as well as positive or negative input from members of the neural tube GRN (Fig. 7).

Shh regulation of the neural tube GRN

Although graded Shh signaling and Gli activity are essential for DV neural tube patterning, the direct targets of Gli regulation in this network are poorly defined. Binding sites for Gli proteins have been investigated in only two Shh-dependent neural tube GRN genes, *Nkx2.2* and *Foxa2*. In both cases, there is evidence that GBSs are required for the expression of these genes in the neural tube (Lei et al., 2006; Sasaki et al., 1997; Vokes et al., 2007). A recent ChIP-on-chip screen has begun to expand the number and location of GBSs (Vokes et al., 2007). A custom array of genomic regions surrounding 122 genes that appear to be regulated by Shh signaling, in neural and non-neural contexts, was screened using Gli1 chromatin immunoprecipitation products from ventralized neural cells, recovering a small number of GBSs, including some close to *Nkx2.2/Nkx2.9* (V3 progenitors) and *Foxa2* (floor plate progenitors). These findings are indicative of, and consistent with, a direct Shh input into localized expression of these determinants (Lei et al., 2006; Sasaki and Hogan, 1996). A whole-genome interrogation is likely to reveal a more complete Gli neural tube GRN. Furthermore, identifying sites where Gli3 binding is lost upon Shh signaling might

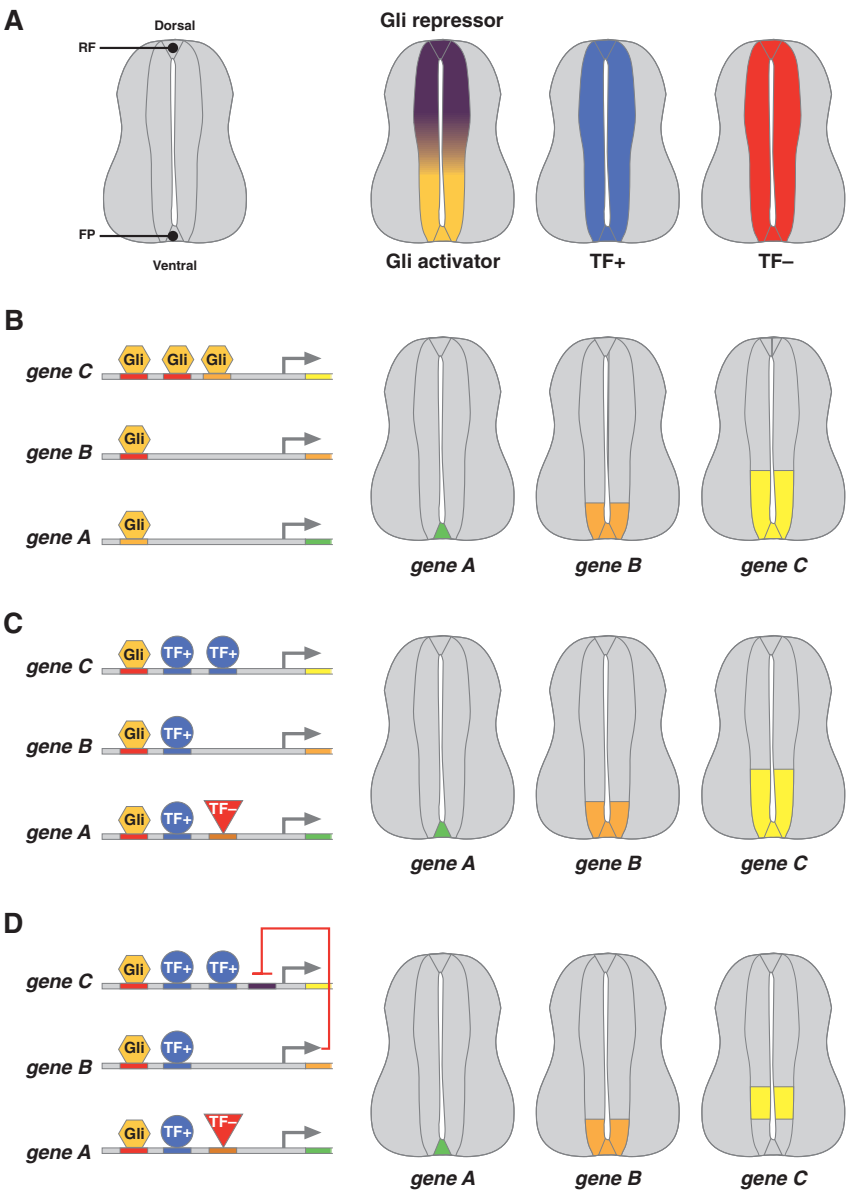

Fig. 8. Potential mechanisms to account for differential response of genes to graded Shh signaling. (**A**) Graded Shh signaling establishes a ventral-to-dorsal gradient of Gli activity (Gli activator, yellow; Gli repressor, purple) in the neural tube. In addition, uniformly expressed activators (blue) and repressors (red) are hypothesized to be expressed in neural progenitors. Three mechanisms could explain the differential sensitivity of genes to the level of Shh signaling: (**B**) The number or affinity of Gli binding sites (GBSs) in a regulatory module might explain differential gene expression. Genes with several high affinity GBSs (yellow gene C) would respond to low concentrations of Shh, resulting in a broad expression domain, with a dorsal boundary far from the ventral midline. Genes with fewer high affinity GBSs (orange gene B) or low affinity GBSs (green gene A) would require higher levels of Gli activity and therefore would be expressed in correspondingly more restricted regions of the neural tube. (**C**) Gli activity may act in conjunction with other repressor and activator signals. In this case, the presence of other transcription factors influences the response of individual genes to Shh signaling. For example, in yellow C and orange gene B, the presence of binding sites for transcription factors acting as activators (TF+) sensitizes the response of these genes, facilitating induction at lower levels of signaling. By contrast, the red gene A, which contains binding sites for a transcriptional inhibitor (TF–), requires higher levels Gli activity and, consequently, higher levels of signaling to overcome the repressive activity. (**D**) The addition of cross-regulation between Shh-dependent genes (see Fig. 7) is likely to refine specific domains of expression in the ventral neural tube. Adding an inhibitory input between two genes from the network in panel C restricts the expression of gene C to a specific domain of progenitors. Such a mechanism could account for the regulation of genes such as *Nkx2.2* and *Olig2*. RF, roof plate; FP, floor plate; Gli, Gli transcription factor; TF, transcription factor.

reveal a different set of targets to those bound by Gli1 activator. The low number of GBSs in the Shh-regulated gene set raises the possibility that only a limited number of genes within the neural tube GRN require a direct Gli input. If so, a direct Shh input might create a 'pioneer' patterning response, while cross-regulatory interactions within the neural tube GRN refine DV pattern through additional transcriptional inputs.

In silico methods to predict GBSs in the genome have also been employed with different degrees of success (Hallikas et al., 2006; Vokes et al., 2007). However, these approaches make significant numbers of false-positive and false-negative predictions. A better understanding of the regulatory logic that underlies the binding and activity of transcription factors will generally facilitate de novo predictions of regulatory mechanisms from primary genomic sequence. These approaches are not able to predict in which target tissue a particular GBS operates. Thus, predictions need to be intersected with other data (e.g. transcriptional profiling) to unravel networks in a specific cell population.

The basic question of how graded Shh signaling is interpreted at the genomic level to control differential gene expression remains to be answered. In other systems, differential gene expression is regulated in response to the positioning, affinity or number of binding sites for a morphogen-regulated transcription factor (Driever et al., 1989; Gaudet and Mango, 2002; Stathopoulos et al., 2002). Genes that contain higher affinity, superior-positioned or increased numbers of binding sites for the relevant factor respond at lower concentration thresholds than do genes with less optimal regulatory inputs (Fig. 8).

A detailed experimental assessment of GBSs in the neural tube GRN genes can address this model in the ventral neural tube. Interestingly, the vertebrate *Ptch1* gene is flanked by several GBSs, suggesting that the presence of multiple sites might contribute to the sensitivity of *Ptch1* expression to Shh signaling (Agren et al., 2004; Vokes et al., 2007). By contrast, *Foxa2* and *Nkx2.2* contain only one clear GBS in a cis-regulatory element that controls ventral neural expression (Lei et al., 2006; Sasaki

and Hogan, 1996; Sasaki et al., 1997; Vokes et al., 2007). Whether the difference in the sequence of the two GBSs explains the difference in *Foxa2* and *Nkx2.2* expression has not been tested, but seems unlikely. Interestingly, both genes are initially activated by Shh signaling with similar temporal and spatial kinetics (Jeong and McMahon, 2005). Thus, some secondary event generates cells that exclusively express each determinant. More generally, it seems likely that the integration of a direct Gli transcriptional input with other inputs (positive and negative) establishes the threshold response for genes in the neural tube GRN (Fig. 8). Thus, the response threshold of a gene is determined by the sum of regulatory inputs on the gene, not solely by the affinity or number of GBSs. This combinatorial mechanism for setting response thresholds is consistent with the identification in neural tube GRN genes of ECRs that contain binding sites for a suite of transcriptional regulators. In this view, the differential integration of positive and negative transcriptional inputs on an individual target gene determines its threshold response to Shh signaling. A similar mechanism has also been suggested for gene regulation by the *Drosophila* Bicoid morphogen (Ochoa-Espinosa and Small, 2006). This regulatory strategy permits mechanisms, such as feed-forward loops (Mangan and Alon, 2003), that would provide an explanation for the dynamic nature of gene expression in the neural tube, as well as the requirement for prolonged duration of signaling for the induction of some neural tube GRN genes.

The regulation of the neural tube GRN

Combined experimental and bioinformatic approaches are beginning to piece together the neural tube GRN, although the details are still fragmentary. Two key ideas emerge. First, specific regulatory interactions between genes in the neural tube GRNs are important for defining the spatio-temporal profiles of gene expression. This aspect of the network is yet to be addressed in detail at the molecular level. Second, the role of graded Shh signaling within the neural tube GRN provides a positional basis to the network by regulating the extent and timing of expression of certain transcription factors within the neural tube GRN. In this view, graded Shh signaling imparts positional information by providing one of a number of inputs into the regulation of members of the neural tube GRN (Fig. 8). The predetermined regulatory interactions within the neural tube GRN transpose this positional information to the tightly regulated patterns of gene expression characteristic of the neural tube. This regulatory strategy might also shed light on the intermixing of cells that express markers of different progenitor domains that is observed in mouse embryos mutant for both *Shh* and *Gli3* or lacking all Gli activity (Bai et al., 2004; Litingtung and Chiang, 2000; Wijgerde et al., 2002). In these embryos, the absence of transcriptional input from Gli proteins removes positional information from the GRN. However, the cross-regulatory connections within the network would be unaffected. Therefore, within individual progenitors, stochastic biases and/or other extrinsic signals present in the neural tube might affect the neural tube GRN. The absence of consistent positional information from Shh-Gli activity would mean that these biases would be dominant. Accordingly, neighboring cells could adopt different positional identities and the neural tube would consist of intermixed cell identities.

A clear priority is to systematically identify new members of the neural tube GRN, to dissect the regulatory mechanisms determining their expression and to elucidate their own regulatory properties. In particular, it will be important to identify the target genes that ultimately determine the subtype identity of neurons generated from each of the progenitor domains. Moreover, an array of cell behaviors, including cell proliferation and cell affinity, is likely to contribute to the precise organization of DV pattern. Regulators of these processes might be uncovered in the neural tube GRN. Finally, although there is a clear divergence in the initiating events, an apparent conservation is evident in neural patterning within vertebrate and invertebrate systems (Chu et al., 1998; Denes et al., 2007; McDonald et al., 1998; Weiss et al., 1998). Thus, it is reasonable to speculate that the neural tube GRN is an evolutionarily conserved, ancestral network that arose in the common ancestor of bilaterally symmetrical animals.

Conclusions

The progress towards understanding DV patterning of the neural tube provides insight into some of the underlying principles that determine the exquisite pattern of vertebrate neurogenesis. What has been revealed is a complex, integrated network of molecular and genetic interactions that receives and interprets the positional information supplied by graded Shh signaling. Importantly, neural target cells are not passive recipients of a graded Shh signal; instead, the cells dynamically influence the spread of the signal through the tissue and alter their response to the signal. This suggests that in place of the conventional view of a morphogen (Kerszberg and Wolpert, 2007), a definition needs to be considered in which the concentration and duration of signaling, together with the response of the tissue, are critical for the formation and interpretation of the morphogen gradient (Box 1). Thus, signal and tissue collaborate to produce a specific pattern. Our understanding will be enhanced if these events can be dynamically imaged and quantified and the complete neural tube GRN defined. A diversity of experimental approaches – genetic, molecular, imaging and modeling – are required to provide a clearer picture of this dynamic, multi-dimensional system.

In this review, we have focused on one crucial component, Shh. However, a similar patterning strategy is thought to underlie neural specification in the dorsal neural tube (Chizhikov and Millen, 2005; Liu and Niswander, 2005). Here, transforming growth factor (TGF) β and Wnt family members are the dominant positional cues, but regulatory interactions between transcription factors controlled by extrinsic and intrinsic signals remain central to the control of positional identity. Furthermore, in addition to their role in the dorsal neural tube, bone morphogenetic protein (BMP) and Wnt signaling also appear to influence patterning in the ventral neural tube (Alvarez-Medina et al., 2008; Lei et al., 2006). How cells integrate the effects of multiple co-incident signals in order to generate an appropriate response will be a crucial question for future studies.

We thank Eva Kutejova for helpful comments. E.D. is supported by EMBO and Marie Curie Fellowships. J.B. is supported by the MRC (UK). Work in A.P.M's laboratory is supported by grants from the NIH.

References

Agren, M., Kogerman, P., Kleman, M. I., Wessling, M. and Toftgard, R. (2004). Expression of the PTCH1 tumor suppressor gene is regulated by alternative promoters and a single functional Gli-binding site. *Gene* **330**, 101-114.

Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M. and Hooper, J. E. (1996). The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell* **86**, 221-232.

Allen, B. L., Tenzen, T. and McMahon, A. P. (2007). The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development. *Genes Dev.* **21**, 1244-1257.

Alvarez-Medina, R., Cayuso, J., Okubo, T., Takada, S. and Marti, E. (2008). Wnt canonical pathway restricts graded Shh/Gli patterning activity through the regulation of Gli3 expression. *Development* **135**, 237-247.

Bai, C. B., Stephen, D. and Joyner, A. L. (2004). All mouse ventral spinal cord

patterning by hedgehog is Gli dependent and involves an activator function of Gli3. *Dev. Cell* **6**, 103-115.
Bailey, P. J., Klos, J. M., Andersson, E., Karlen, M., Kallstrom, M., Ponjavic, J., Muhr, J., Lenhard, B., Sandelin, A. and Ericson, J. (2006). A global genomic transcriptional code associated with CNS-expressed genes. *Exp. Cell Res.* **312**, 3108-3119.
Bijlsma, M. F., Spek, C. A., Zivkovic, D., van de Water, S., Rezaee, F. and Peppelenbosch, M. P. (2006). Repression of smoothened by patched-dependent (pro-)vitamin D3 secretion. *PLoS Biol.* **4**, e232.
Bourikas, D., Pekarik, V., Baeriswyl, T., Grunditz, A., Sadhu, R., Nardo, M. and Stoeckli, E. T. (2005). Sonic hedgehog guides commissural axons along the longitudinal axis of the spinal cord. *Nat. Neurosci.* **8**, 297-304.
Briscoe, J. and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr. Opin. Neurobiol.* **11**, 43-49.
Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T. M., Rubenstein, J. L. and Ericson, J. (1999). Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* **398**, 622-627.
Briscoe, J., Chen, Y., Jessell, T. M. and Struhl, G. (2001). A hedgehog-insensitive form of patched provides evidence for direct long-range morphogen activity of sonic hedgehog in the neural tube. *Mol. Cell* **7**, 1279-1291.
Bumcrot, D. A., Takada, R. and McMahon, A. P. (1995). Proteolytic processing yields two secreted forms of sonic hedgehog. *Mol. Cell. Biol.* **15**, 2294-2303.
Caspary, T., Garcia-Garcia, M. J., Huangfu, D., Eggenschwiler, J. T., Wyler, M. R., Rakeman, A. S., Alcorn, H. L. and Anderson, K. V. (2002). Mouse Dispatched homolog1 is required for long-range, but not juxtacrine, Hh signaling. *Curr. Biol.* **12**, 1628-1632.
Chamberlain, C. E., Jeong, J., Guo, C., Allen, B. L. and McMahon, A. P. (2008). Notochord-derived Shh concentrates in close association with the apically positioned basal body in neural target cells and forms a dynamic gradient during neural patterning. *Development* **135**, 1097-1106.
Charron, F., Stein, E., Jeong, J., McMahon, A. P. and Tessier-Lavigne, M. (2003). The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. *Cell* **113**, 11-23.
Chen, J. K., Taipale, J., Cooper, M. K. and Beachy, P. A. (2002a). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev.* **16**, 2743-2748.
Chen, J. K., Taipale, J., Young, K. E., Maiti, T. and Beachy, P. A. (2002b). Small molecule modulation of Smoothened activity. *Proc. Natl. Acad. Sci. USA* **99**, 14071-14076.
Chen, M. H., Li, Y. J., Kawakami, T., Xu, S. M. and Chuang, P. T. (2004). Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. *Genes Dev.* **18**, 641-659.
Chizhikov, V. V. and Millen, K. J. (2005). Roof plate-dependent patterning of the vertebrate dorsal central nervous system. *Dev. Biol.* **277**, 287-295.
Chu, H., Parras, C., White, K. and Jimenez, F. (1998). Formation and specification of ventral neuroblasts is controlled by vnd in Drosophila neurogenesis. *Genes Dev.* **12**, 3613-3624.
Chuang, P. T. and McMahon, A. P. (1999). Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. *Nature* **397**, 617-621.
Chuang, P. T., Kawcak, T. and McMahon, A. P. (2003). Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung. *Genes Dev.* **17**, 342-347.
Corbit, K. C., Aanstad, P., Singla, V., Norman, A. R., Stainier, D. Y. and Reiter, J. F. (2005). Vertebrate Smoothened functions at the primary cilium. *Nature* **437**, 1018-1021.
Corcoran, R. B. and Scott, M. P. (2006). Oxysterols stimulate Sonic hedgehog signal transduction and proliferation of medulloblastoma cells. *Proc. Natl. Acad. Sci. USA* **103**, 8408-8413.
Danesin, C., Agius, E., Escalas, N., Ai, X., Emerson, C., Cochard, P. and Soula, C. (2006). Ventral neural progenitors switch toward an oligodendroglial fate in response to increased Sonic hedgehog (Shh) activity: involvement of Sulfatase 1 in modulating Shh signaling in the ventral spinal cord. *J. Neurosci.* **26**, 5037-5048.
Denes, A. S., Jekely, G., Steinmetz, P. R., Raible, F., Snyman, H., Prud'homme, B., Ferrier, D. E., Balavoine, G. and Arendt, D. (2007). Molecular architecture of annelid nerve cord supports common origin of nervous system centralization in bilateria. *Cell* **129**, 277-288.
Dessaud, E., Yang, L. L., Hill, K., Cox, B., Ulloa, F., Ribeiro, A., Mynett, A., Novitch, B. G. and Briscoe, J. (2007). Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism. *Nature* **450**, 717-720.
Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J. and Hui, C. C. (1998). Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. *Development* **125**, 2533-2543.
Driever, W., Thoma, G. and Nusslein-Volhard, C. (1989). Determination of spatial domains of zygotic gene expression in the Drosophila embryo by the affinity of binding sites for the bicoid morphogen. *Nature* **340**, 363-367.
Dwyer, J. R., Sever, N., Carlson, M., Nelson, S. F., Beachy, P. A. and Parhami, F. (2007). Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells. *J. Biol. Chem.* **282**, 8959-8968.
Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.
Eggenschwiler, J. T. and Anderson, K. V. (2007). Cilia and developmental signaling. *Annu. Rev. Cell Dev. Biol.* **23**, 345-373.
Eldar, A., Rosin, D., Shilo, B. Z. and Barkai, N. (2003). Self-enhanced ligand degradation underlies robustness of morphogen gradients. *Dev. Cell* **5**, 635-646.
Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.
Frank-Kamenetsky, M., Zhang, X. M., Bottega, S., Guicherit, O., Wichterle, H., Dudek, H., Bumcrot, D., Wang, F. Y., Jones, S., Shulok, J. et al. (2002). Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. *J. Biol.* **1**, 10.
Gaudet, J. and Mango, S. E. (2002). Regulation of organogenesis by the Caenorhabditis elegans FoxA protein PHA-4. *Science* **295**, 821-825.
Goetz, J. A., Singh, S., Suber, L. M., Kull, F. J. and Robbins, D. J. (2006). A highly conserved amino-terminal region of sonic hedgehog is required for the formation of its freely diffusible multimeric form. *J. Biol. Chem.* **281**, 4087-4093.
Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A. and Scott, M. P. (1996). Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev.* **10**, 301-312.
Goodrich, L. V., Milenkovic, L., Higgins, K. M. and Scott, M. P. (1997). Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* **277**, 1109-1113.
Gritli-Linde, A., Lewis, P., McMahon, A. P. and Linde, A. (2001). The whereabouts of a morphogen: direct evidence for short- and graded long-range activity of hedgehog signaling peptides. *Dev. Biol.* **236**, 364-386.
Hallikas, O., Palin, K., Sinjushina, N., Rautiainen, R., Partanen, J., Ukkonen, E. and Taipale, J. (2006). Genome-wide prediction of mammalian enhancers based on analysis of transcription-factor binding affinity. *Cell* **124**, 47-59.
Haycraft, C. J., Banizs, B., Aydin-Son, Y., Zhang, Q., Michaud, E. J. and Yoder, B. K. (2005). Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. *PLoS Genet.* **1**, e53.
Hochstim, C., Deneen, B., Lukaszewicz, A., Zhou, Q. and Anderson, D. J. (2008). Identification of positionally distinct astrocyte subtypes whose identities are specified by a homeodomain code. *Cell* **133**, 510-522.
Hollway, G. E., Maule, J., Gautier, P., Evans, T. M., Keenan, D. G., Lohs, C., Fischer, D., Wicking, C. and Currie, P. D. (2006). Scube2 mediates Hedgehog signalling in the zebrafish embryo. *Dev. Biol.* **294**, 104-118.
Hooper, J. E. (2003). Smoothened translates Hedgehog levels into distinct responses. *Development* **130**, 3951-3963.
Huang, X., Litingtung, Y. and Chiang, C. (2007). Region-specific requirement for cholesterol modification of sonic hedgehog in patterning the telencephalon and spinal cord. *Development* **134**, 2095-2105.
Huangfu, D. and Anderson, K. V. (2006). Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from Drosophila to vertebrates. *Development* **133**, 3-14.
Incardona, J. P., Gruenberg, J. and Roelink, H. (2002). Sonic hedgehog induces the segregation of patched and smoothened in endosomes. *Curr. Biol.* **12**, 983-995.
Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.
Jacob, J. and Briscoe, J. (2003). Gli proteins and the control of spinal-cord patterning. *EMBO Rep.* **4**, 761-765.
Jaeger, J. and Reinitz, J. (2006). On the dynamic nature of positional information. *BioEssays* **28**, 1102-1111.
Jeong, J. and McMahon, A. P. (2005). Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1. *Development* **132**, 143-154.
Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.
Jia, J., Tong, C. and Jiang, J. (2003). Smoothened transduces Hedgehog signal by physically interacting with Costal2/Fused complex through its C-terminal tail. *Genes Dev.* **17**, 2709-2720.
Jia, J., Tong, C., Wang, B., Luo, L. and Jiang, J. (2004). Hedgehog signalling activity of Smoothened requires phosphorylation by protein kinase A and casein kinase I. *Nature* **432**, 1045-1050.
Katoh, Y. and Katoh, M. (2004a). Characterization of KIF7 gene in silico. *Int. J. Oncol.* **25**, 1881-1886.
Katoh, Y. and Katoh, M. (2004b). KIF27 is one of orthologs for Drosophila Costal-2. *Int. J. Oncol.* **25**, 1875-1880.
Kawakami, T., Kawcak, T., Li, Y. J., Zhang, W., Hu, Y. and Chuang, P. T. (2002).

Mouse dispatched mutants fail to distribute hedgehog proteins and are defective in hedgehog signaling. *Development* **129**, 5753-5765.
Kawakami, A., Nojima, Y., Toyoda, A., Takahoko, M., Satoh, M., Tanaka, H., Wada, H., Masai, I., Terasaki, H., Sakaki, Y. et al. (2005). The zebrafish-secreted matrix protein you/scube2 is implicated in long-range regulation of hedgehog signaling. *Curr. Biol.* **15**, 480-488.
Kerszberg, M. and Wolpert, L. (2007). Specifying positional information in the embryo: looking beyond morphogens. *Cell* **130**, 205-209.
Lee, J. J., Ekker, S. C., von Kessler, D. P., Porter, J. A., Sun, B. I. and Beachy, P. A. (1994). Autoproteolysis in hedgehog protein biogenesis. *Science* **266**, 1528-1537.
Lei, Q., Zelman, A. K., Kuang, E., Li, S. and Matise, M. P. (2004). Transduction of graded Hedgehog signaling by a combination of Gli2 and Gli3 activator functions in the developing spinal cord. *Development* **131**, 3593-3604.
Lei, Q., Jeong, Y., Misra, K., Li, S., Zelman, A. K., Epstein, D. J. and Matise, M. P. (2006). Wnt signaling inhibitors regulate the transcriptional response to morphogenetic Shh-Gli signaling in the neural tube. *Dev. Cell* **11**, 325-337.
Litingtung, Y. and Chiang, C. (2000). Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. *Nat. Neurosci.* **3**, 979-985.
Liu, A. and Niswander, L. A. (2005). Bone morphogenetic protein signalling and vertebrate nervous system development. *Nat. Rev. Neurosci.* **6**, 945-954.
Lum, L., Zhang, C., Oh, S., Mann, R. K., von Kessler, D. P., Taipale, J., Weis-Garcia, F., Gong, R., Wang, B. and Beachy, P. A. (2003). Hedgehog signal transduction via Smoothened association with a cytoplasmic complex scaffolded by the atypical kinesin, Costal-2. *Mol. Cell* **12**, 1261-1274.
Lupo, G., Harris, W. A. and Lewis, K. E. (2006). Mechanisms of ventral patterning in the vertebrate nervous system. *Nat. Rev. Neurosci.* **7**, 103-114.
Ma, Y., Erkner, A., Gong, R., Yao, S., Taipale, J., Basler, K. and Beachy, P. A. (2002). Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched. *Cell* **111**, 63-75.
Mangan, S. and Alon, U. (2003). Structure and function of the feed-forward loop network motif. *Proc. Natl. Acad. Sci. USA* **100**, 11980-11985.
Marigo, V. and Tabin, C. J. (1996). Regulation of patched by sonic hedgehog in the developing neural tube. *Proc. Natl. Acad. Sci. USA* **93**, 9346-9351.
Marti, E., Takada, R., Bumcrot, D. A., Sasaki, H. and McMahon, A. P. (1995). Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. *Development* **121**, 2537-2547.
Martinelli, D. C. and Fan, C. M. (2007). The role of Gas1 in embryonic development and its implications for human disease. *Cell Cycle* **6**, 2650-2655.
Matise, M. P. and Joyner, A. L. (1999). Gli genes in development and cancer. *Oncogene* **18**, 7852-7859.
Matise, M. P., Lustig, M., Sakurai, T., Grumet, M. and Joyner, A. L. (1999). Ventral midline cells are required for the local control of commissural axon guidance in the mouse spinal cord. *Development* **126**, 3649-3659.
McDonald, J. A., Holbrook, S., Isshiki, T., Weiss, J., Doe, C. Q. and Mellerick, D. M. (1998). Dorsoventral patterning in the Drosophila central nervous system: the vnd homeobox gene specifies ventral column identity. *Genes Dev.* **12**, 3603-3612.
Michaux, G., Gansmuller, A., Hindelang, C. and Labouesse, M. (2000). CHE-14, a protein with a sterol-sensing domain, is required for apical sorting in C. elegans ectodermal epithelial cells. *Curr. Biol.* **10**, 1098-1107.
Muhr, J., Andersson, E., Persson, M., Jessell, T. M. and Ericson, J. (2001). Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube. *Cell* **104**, 861-873.
Novitch, B. G., Chen, A. I. and Jessell, T. M. (2001). Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. *Neuron* **31**, 773-789.
Ochoa-Espinosa, A. and Small, S. (2006). Developmental mechanisms and cis-regulatory codes. *Curr. Opin. Genet. Dev.* **16**, 165-170.
Okada, A., Charron, F., Morin, S., Shin, D. S., Wong, K., Fabre, P. J., Tessier-Lavigne, M. and McConnell, S. K. (2006). Boc is a receptor for sonic hedgehog in the guidance of commissural axons. *Nature* **444**, 369-373.
Pages, F. and Kerridge, S. (2000). Morphogen gradients. A question of time or concentration? *Trends Genet.* **16**, 40-44.
Pan, Y., Bai, C. B., Joyner, A. L. and Wang, B. (2006). Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. *Mol. Cell. Biol.* **26**, 3365-3377.
Park, H. L., Bai, C., Platt, K. A., Matise, M. P., Beeghly, A., Hui, C. C., Nakashima, M. and Joyner, A. L. (2000). Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. *Development* **127**, 1593-1605.
Pepinsky, R. B., Zeng, C., Wen, D., Rayhorn, P., Baker, D. P., Williams, K. P., Bixler, S. A., Ambrose, C. M., Garber, E. A., Miatkowski, K. et al. (1998). Identification of a palmitic acid-modified form of human Sonic hedgehog. *J. Biol. Chem.* **273**, 14037-14045.
Persson, M., Stamataki, D., te Welscher, P., Andersson, E., Bose, J., Ruther, U., Ericson, J. and Briscoe, J. (2002). Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity. *Genes Dev.* **16**, 2865-2878.
Placzek, M. and Briscoe, J. (2005). The floor plate: multiple cells, multiple signals. *Nat. Rev. Neurosci.* **6**, 230-240.
Porter, J. A., von Kessler, D. P., Ekker, S. C., Young, K. E., Lee, J. J., Moses, K. and Beachy, P. A. (1995). The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. *Nature* **374**, 363-366.
Porter, J. A., Ekker, S. C., Park, W. J., von Kessler, D. P., Young, K. E., Chen, C. H., Ma, Y., Woods, A. S., Cotter, R. J., Koonin, E. V. et al. (1996a). Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. *Cell* **86**, 21-34.
Porter, J. A., Young, K. E. and Beachy, P. A. (1996b). Cholesterol modification of hedgehog signaling proteins in animal development. *Science* **274**, 255-259.
Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. et al. (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76**, 761-775.
Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.
Rohatgi, R., Milenkovic, L. and Scott, M. P. (2007). Patched1 regulates hedgehog signaling at the primary cilium. *Science* **317**, 372-376.
Rubin, J. B., Choi, Y. and Segal, R. A. (2002). Cerebellar proteoglycans regulate sonic hedgehog responses during development. *Development* **129**, 2223-2232.
Ruel, L., Rodriguez, R., Gallet, A., Lavenant-Staccini, L. and Therond, P. P. (2003). Stability and association of Smoothened, Costal2 and Fused with Cubitus interruptus are regulated by Hedgehog. *Nat. Cell Biol.* **5**, 907-913.
Saha, K. and Schaffer, D. V. (2006). Signal dynamics in Sonic hedgehog tissue patterning. *Development* **133**, 889-900.
Sasaki, H. and Hogan, B. L. (1996). Enhancer analysis of the mouse HNF-3 beta gene: regulatory elements for node/notochord and floor plate are independent and consist of multiple sub-elements. *Genes Cells* **1**, 59-72.
Sasaki, H., Hui, C., Nakafuku, M. and Kondoh, H. (1997). A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313-1322.
Sheng, T., Chi, S., Zhang, X. and Xie, J. (2006). Regulation of Gli1 localization by the cAMP/protein kinase A signaling axis through a site near the nuclear localization signal. *J. Biol. Chem.* **281**, 9-12.
Stamataki, D., Ulloa, F., Tsoni, S. V., Mynett, A. and Briscoe, J. (2005). A gradient of Gli activity mediates graded Sonic Hedgehog signaling in the neural tube. *Genes Dev.* **19**, 626-641.
Stathopoulos, A., Van Drenth, M., Erives, A., Markstein, M. and Levine, M. (2002). Whole-genome analysis of dorsal-ventral patterning in the Drosophila embryo. *Cell* **111**, 687-701.
Svard, J., Heby-Henricson, K., Persson-Lek, M., Rozell, B., Lauth, M., Bergstrom, A., Ericson, J., Toftgard, R. and Teglund, S. (2006). Genetic elimination of Suppressor of fused reveals an essential repressor function in the mammalian Hedgehog signaling pathway. *Dev. Cell* **10**, 187-197.
Tay, S. Y., Ingham, P. W. and Roy, S. (2005). A homologue of the Drosophila kinesin-like protein Costal2 regulates Hedgehog signal transduction in the vertebrate embryo. *Development* **132**, 625-634.
Taylor, F. R., Wen, D., Garber, E. A., Carmillo, A. N., Baker, D. P., Arduini, R. M., Williams, K. P., Weinreb, P. H., Rayhorn, P., Hronowski, X. et al. (2001). Enhanced potency of human Sonic hedgehog by hydrophobic modification. *Biochemistry* **40**, 4359-4371.
Tenzen, T., Allen, B. L., Cole, F., Kang, J. S., Krauss, R. S. and McMahon, A. P. (2006). The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice. *Dev. Cell* **10**, 647-656.
Tian, H., Jeong, J., Harfe, B. D., Tabin, C. J. and McMahon, A. P. (2005). Mouse Disp1 is required in sonic hedgehog-expressing cells for paracrine activity of the cholesterol-modified ligand. *Development* **132**, 133-142.
Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T. M. and Ericson, J. (2001). Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. *Neuron* **31**, 743-755.
Varjosalo, M., Li, S. P. and Taipale, J. (2006). Divergence of hedgehog signal transduction mechanism between Drosophila and mammals. *Dev. Cell* **10**, 177-186.
Vokes, S. A., Ji, H., McCuine, S., Tenzen, T., Giles, S., Zhong, S., Longabaugh, W. J., Davidson, E. H., Wong, W. H. and McMahon, A. P. (2007). Genomic characterization of Gli-activator targets in sonic hedgehog-mediated neural patterning. *Development* **134**, 1977-1989.
Weiss, J. B., Von Ohlen, T., Mellerick, D. M., Dressler, G., Doe, C. Q. and Scott, M. P. (1998). Dorsoventral patterning in the Drosophila central nervous system: the intermediate neuroblasts defective homeobox gene specifies intermediate column identity. *Genes Dev.* **12**, 3591-3602.
Wijgerde, M., McMahon, J. A., Rule, M. and McMahon, A. P. (2002). A direct requirement for Hedgehog signaling for normal specification of all ventral

progenitor domains in the presumptive mammalian spinal cord. *Genes Dev.* **16**, 2849-2864.

Williams, K. P., Rayhorn, P., Chi-Rosso, G., Garber, E. A., Strauch, K. L., Horan, G. S., Reilly, J. O., Baker, D. P., Taylor, F. R., Koteliansky, V. et al. (1999). Functional antagonists of sonic hedgehog reveal the importance of the N terminus for activity. *J. Cell Sci.* **112**, 4405-4414.

Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. *J. Theor. Biol.* **25**, 1-47.

Wolpert, L. (1996). One hundred years of positional information. *Trends Genet.* **12**, 359-364.

Yao, S., Lum, L. and Beachy, P. (2006). The ihog cell-surface proteins bind Hedgehog and mediate pathway activation. *Cell* **125**, 343-357.

Zeng, X., Goetz, J. A., Suber, L. M., Scott, W. J., Jr, Schreiner, C. M. and Robbins, D. J. (2001). A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature* **411**, 716-720.

Zhang, W., Kang, J. S., Cole, F., Yi, M. J. and Krauss, R. S. (2006). Cdo functions at multiple points in the Sonic Hedgehog pathway, and Cdo-deficient mice accurately model human holoprosencephaly. *Dev. Cell* **10**, 657-665.

Zhang, X. M., Ramalho-Santos, M. and McMahon, A. P. (2001). Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R symmetry by the mouse node. *Cell* **106**, 781-792.

Zhao, Y., Tong, C. and Jiang, J. (2007). Hedgehog regulates smoothened activity by inducing a conformational switch. *Nature* **450**, 252-258.