

From CELL AND MOLECULAR BIOLOGY  
Karolinska Institutet, Stockholm, Sweden

# **MORPHOGEN INTERPRETATION IN THE DEVELOPING NERVOUS SYSTEM**

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Picture on the cover depicts the expression of Shh protein in the HH16 chick spinal cord.

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*To the generations of strong women in my family*



## ABSTRACT

Development of the central nervous system relies on the generation of specialized cell types in a tightly controlled spatial and temporal order from neural progenitor cells. Morphogen molecules, secreted by defined sources, spatially organize neural progenitors by inducing discrete expression patterns of cell fate determinant genes in a concentration-dependent manner. The combinatorial expression of these patterning genes defines distinct progenitor domains from which specific neuronal subtypes are generated. This thesis deals with one of the big challenges in developmental sciences, which is to understand how these inductive gradients are translated into precise transcriptional outputs.

Sonic Hedgehog (Shh) is a morphogen essential for the generation of ventral neuronal subtypes. In **Paper I**, we have identified the *cis*-regulatory modules (CRM) of neural Shh-target genes, which we use as tools to elucidate the mechanisms imposed by Gli proteins, the bifunctional transcriptional mediators of Shh gradient. We find that Gli activators have a non-instructive role in long-range patterning and in synergy with SoxB1 proteins activate Shh target genes in a largely concentration independent manner. Instead, Gli repressors are interpreted at transcriptional levels into precise spatial gene patterns in combination with regional homeodomain co-repressors. Moreover, the local interpretation of Shh displays lower CRM context sensitivity and requires Gli activators to accumulate to a threshold level sufficient to counteract Gli repressors. Thus our data propose a novel mechanism for transcriptional interpretation of Shh gradient.

**Paper II** studies a feedback circuit between Shh and its downstream homeodomain targets that establishes the non-graded regulation of Shh signaling activity. We show that by regulating *Gli3* expression, Nkx2 proteins amplify and Pax6 antagonizes Shh signaling. The amplified Shh response is important for specification of the two most ventral cell fates: the floor plate (FP) and V3. However, the spatial separation of the two domains appears to be achieved by the acquisition of neurogenic potential over time in the p3 domain, rather than by different Shh concentrations. These data establish that the non-graded, intrinsic changes in responding cells operate in parallel with graded mechanisms and are required for correct interpretation of Shh signaling.

Morphogens are pleiotropic signals that regulate development of various tissues, but how they induce tissue-specific responses remains unresolved. **Paper III** explores the tissue-specific interpretation of Shh, Bone Morphogenic Proteins (BMP) and Retinoic Acid (RA) signaling and shows that direct transcriptional integration of these pathways with SoxB1 proteins at the CRM level is required for activation of neural targets. We further show that the genome-wide collocation of binding sites for SoxB1 and morphogen-mediatory transcription factors in CRMs can faithfully predict the neural-specific gene activity. Moreover, misexpression of SoxB1 proteins in the limb bud confers mesodermal cells with the potential to activate neural-specific target genes upon activation of Shh, BMP or RA signaling. Accordingly, our data offers a fairly simple conceptual explanation for morphogen-mediated transcriptional regulation of neural-specific target genes during embryogenesis.



## LIST OF PUBLICATIONS

This thesis is based on following articles, which will be referred to in the text according to their roman numerals:

- I.** Oosterveen T\*, **Kurdija S\***, Alekseenko Z, Uhde CW, Bergsland M, Sandberg M, Andersson E, Dias JM, Muhr J, Ericson J, (2012).  
Mechanistic differences in the transcriptional interpretation of local and long-range shh morphogen signaling.  
Developmental Cell 23(5):1006-19.
- II.** Lek M\*, Dias JM\*, Marklund U, Uhde CW, **Kurdija S**, Lei Q, Sussel L, Rubenstein JL, Matise MP, Arnold HH, Jessell TM, Ericson J. (2010)  
A homeodomain feedback circuit underlies step-function interpretation of a Shh morphogen gradient during ventral neural patterning.  
Development 137(23):4051-60
- III.** Oosterveen T\*, **Kurdija S\***, Ensterö M, Uhde CW, Bergsland M, Sandberg M, Sandberg R, Muhr J and Ericson J  
A SoxB1-driven transcriptional network underlies neural-specific interpretation of morphogen signals.  
Submitted

\* These authors contributed equally





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## LIST OF ABBREVIATIONS

|       |                                  |
|-------|----------------------------------|
| A/P   | Anterior/Posterior               |
| BMP   | Bone Morphogenic Protein         |
| bHLH  | basic Helix Loop Helix           |
| bp    | Base Pair                        |
| CNS   | Central Nervous System           |
| CRM   | cis-Regulatory Module            |
| D/V   | Dorsal/Ventral                   |
| FP    | Floor Plate                      |
| GBS   | Gli Binding Site                 |
| HBS   | Homeo domain Binding Site        |
| HD    | Homeo Domain                     |
| NRBS  | Nuclear Receptor Binding Site    |
| SBS   | SoxB1 Binding Site               |
| Shh   | Sonic Hedgehog                   |
| SmBS  | Smad Binding Sites               |
| SoxB1 | Sox1, Sox2 and Sox3              |
| TF    | Transcription Factor             |
| RA    | Retinoic Acid                    |
| RARE  | Retinoic Acid Responsive Element |
| R/C   | Rostral/Caudal                   |
| RP    | Roof Plate                       |
| ZPA   | Zone of Polarizing Activity      |

# **1. INTRODUCTION**

## **1.1 The complexity of the CNS**

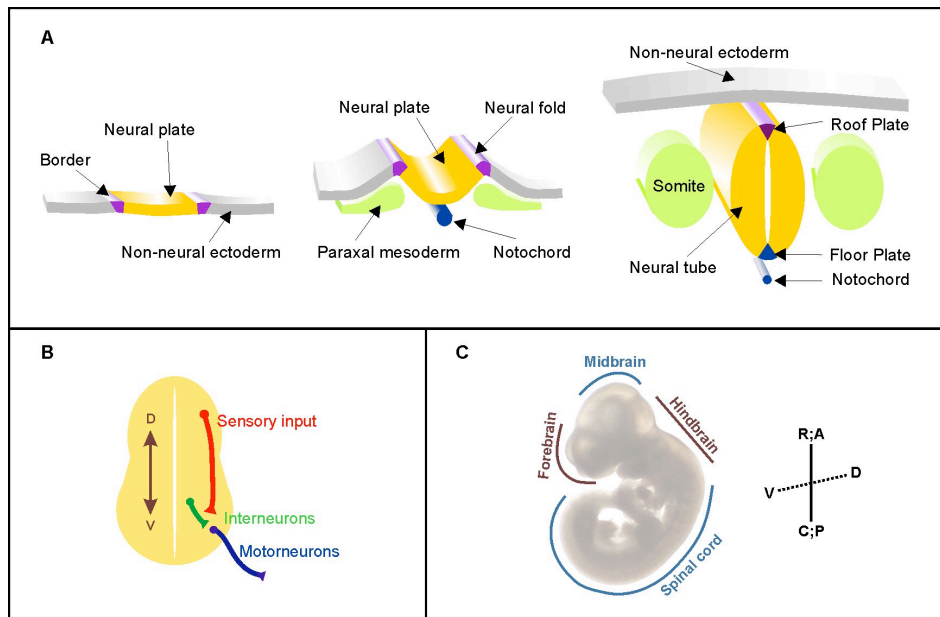
The central nervous system (CNS) is a complex organ that consists of approximately 10 billion neurons and additional 100 billion macroglial cells (adult human brain) (Williams and Herrup, 1988). Neurons are organized into complex networks that process and convey information between the brain and peripheral organs in the form of electrical impulses, a function which allows for the regulation of our basic body functions and cognitive abilities. The neurons can be subdivided into thousands of distinct neuronal subtypes, which have unique morphological and signaling properties and comprise specific parts of neuronal circuits. In addition, there are two major types of macroglial cells: oligodendrocytes and astrocytes. Oligodendrocytes are myelin producing cells that insulate neuronal axons to allow fast conduction of electrical impulses, whereas astrocytes provide structural support and regulate chemical balance (Rowitch and Kriegstein, 2010). This large variety of specialized cell types originates during early embryogenesis and is organized into a functional organ that forms the essence of animal life.

### **1.1.1. Early neural development**

The vertebrate organism develops from the stem cells of the three germ layers, ectoderm, mesoderm and endoderm through successive, tightly controlled spatial and temporal conversions of progenitor fates, where each step has progressively lower stem cell potential. CNS progenitors derive from the naïve ectoderm through actions of signaling molecules such as Fibroblast Growth Factors and Bone Morphogenic Protein inhibitors and appear as a sheet of cells called the neural plate (Figure 1A) (Sadler, 2005; Stern, 2006). By contrast, the ectodermal cells that do not receive these signals develop into epidermal progenitors giving rise to skin. At the time of neural plate induction, neural progenitors have the potential to generate most specialized cells of the CNS; however, as development proceeds this potential becomes gradually restricted (Gabel, 2012; Kriegstein and Alvarez-Buylla, 2009).

Subsequently, the elongation and thickening of the neural plate cells and the series of cell migrations in the underlying mesoderm and lateral ectoderm cause the neural plate to invaginate and fold in a process called neurulation (Figure 1A). At the end of neurulation, the dorsolateral edges of the neural folds fuse and segregate from non-neural ectoderm thus forming the neural tube (Figure 1A) (Colas and Schoenwolf, 2001; Sadler, 2005). At this stage in development the individual progenitor cells have acquired distinct properties that depend on their spatial position along rostral/caudal (R/C) and dorsal/ventral (D/V) axis of the neural tube (Figure 1B, 1C and 2A), and are destined to generate specific sets of neuronal subtypes.

Along the R/C axis different compartments of the neural tube will develop into discrete structures of the CNS. The rostral-most part develops into the forebrain adjoining the more posterior midbrain, which is followed by the presumptive hindbrain, whereas the posterior part of the neural tube generates the presumptive spinal cord (Figure 1C). The forebrain becomes further subdivided into cerebrum and diencephalon while the hindbrain develops into the various structures of the brain stem.



**Figure1. The development of the CNS.**

*A) Neural progenitors originate from ectoderm as a sheet of cells called neural plate. As development proceeds, movements of axial and paraxial mesoderm cells, which later develop into notochord and somites, cause the folding of neural plate. Eventually neural folds fuse and separate from the overlying non-neuronal ectoderm, thus forming the neural tube. The dorsalmost and ventralmost cells of the neural tube differentiate into the roof plate and the floor plate, respectively, and function as signaling centers in addition to the notochord and non-neural ectoderm. Somites will later develop into vertebrae and muscles. B) Subsequently, the posterior region of the neural tube matures into the spinal cord, where cells in the dorsal (D) region develop into interneurons that process sensory information and cells in the ventral (V) region mature into interneurons and motorneurons that control body movements. C) Mouse embryo (E11) where large subdivisions of the CNS are indicated. Rostral;Anterior (R;A) – Caudal;Posterior (C;P) and Dorsal (D) – Ventral (V) axes in relationship to the embryo are indicated.*

Along the D/V axis, in spatially distinctive domains, discrete neuronal subtypes are specified. At spinal cord levels, motor neurons, which convey information to muscles, and distinct types of interneurons, which regulate the activity of motor neurons or gather and relay sensory information, are generated (Figure 1B and 2). Later in the development, the glial cells will develop from the same domains (Rowitch and Kriegstein, 2010). As the development advances, all generated cells mature and migrate to their final positions, send projections and connect to their targets and form a functional and robust communication network.

What mechanisms regulate the generation of this vast cellular diversity from early progenitors? This question has intrigued the developmental community during decades

leading to the identification of a multitude of mechanisms involved in cell proliferation, specification, differentiation, migration and apoptosis. The work presented in this thesis sheds light on a part of this intermixed assembly of processes, specifically how the precise pattern of neuronal progenitor identities is established along the D/V axis in the developing spinal cord.

## **1.2. Regulation of cell patterning by morphogens**

Signaling molecules secreted by defined areas of the developing embryo organize surrounding tissues and govern progenitor cell identities. These signaling molecules are considered morphogens if the molecule diffuses away from the source, creating a concentration gradient across few cell diameters and it elicits different intracellular effects in a concentration-dependent manner (Lander, 2007). Mechanistically, morphogens activate specific sets of genes at different concentration thresholds, thus creating spatial gene expression patterns that define distinct progenitor pools and ensure the robust and organized generation of specialized cells in the developing organism.

### **1.2.1. Expression patterns in the developing spinal cord**

The spinal cord is organized in a relatively simple pattern and serves as a model for studying mechanisms of morphogen activity during neural development. The spinal cord contains two sources of morphogen production: the floor plate (FP) located at the ventral midline and the roof plate (RP) located at the dorsal midline, (Figure 1A). The FP and underlying notochord, a rod-like structure consisting of mesodermal cells, secrete Sonic Hedgehog (Shh) protein, whereas the RP and overlying ectoderm produce Bone Morphogenic Proteins (BMP) (Figure 1A and 2A). In addition, retinoic acid (RA) diffuses from the paraxial mesoderm and later from the developing somites (Ulloa and Briscoe, 2007).

Shh, BMP and RA regulate the spatial expression of transcription factors (TF) belonging to the Homeodomain (HD) and basic-Helix-Loop-Helix (bHLH) families (Figure 2B). By expressing unique combinations of these patterning TFs, 11 distinct progenitor domains are established along the D/V axis of the spinal cord. The combinatorial activity of HD and bHLH proteins in each progenitor domain triggers the activation of discrete transcriptional programs that specify distinct neuronal subtypes and ensure correct maturation of differentiated neuronal cells (Ericson et al., 1997a; Jessell, 2000). Thus, each of the 6 dorsal (dp6-dp1) and the 4 ventral (p0-p2, p3) progenitor domains generate specific types of interneurons, whereas the ventral pMN domain generates motor neurons. At later embryonic stages, neural progenitors will switch to production of glial precursors such that the pMN domain will yield oligodendrocytes, while p1, p2 and p3 domains will produce astrocytes (Rowitch and Kriegstein, 2010). Thereby, the large variety of specialized CNS cells is generated and specified in the precise spatial and temporal pattern.

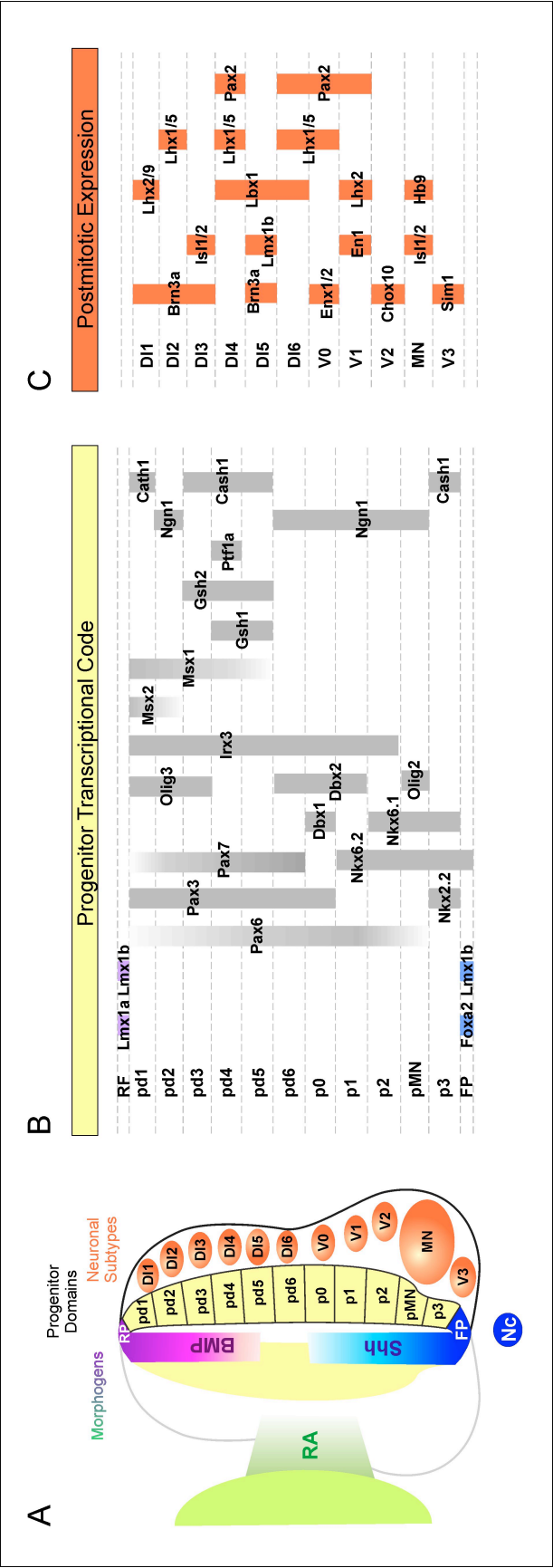


Figure 2. Patterning of the chick spinal cord.

A) BMP secreted by the roof plate (RP), Shh by the floor plate (FP) and notochord and RA by the somites, all regulate the segregation of 11 distinct progenitor domains that will generate distinct early neuronal sub-types. B) The morphogens control spatial expression of HD and bHLH genes that further regulate neuronal subtype specification. Each progenitor domain is thus defined by unique combinatorial expression profile of these transcription factors. C) The generated post-mitotic neurons are defined by expression of various postmitotic markers.

### Cross-repression

Many of the patterning HD and bHLH proteins function as transcriptional repressors and many form selective cross-repressive interactions with another of these TFs, a mechanism that confines their expression into mutually exclusive domains (Figure 3 depicts the cross-repressive interactions in the ventral spinal cord) (Muhr et al., 2001). In addition to repressing alternative subtype differentiation programs, the cross-repressive interactions reinforce the boundaries of adjacent progenitor domains thereby refining and maintaining progenitor domain identities.

### Shh imparts positional identity to progenitors in the ventral spinal cord

In the ventral spinal cord, Shh plays a critical role in orchestrating the patterned expression of HD and bHLH genes. Depending on whether they are repressed or induced by Shh, these patterning genes are grouped into class I or class II, respectively (Figure 3). Two- to three-fold increases of Shh recombinant protein are sufficient to sequentially induce progressively more ventral class II genes in naïve neural progenitors explanted from the chick intermediate neural tube (Briscoe et al., 2000; Roelink et al., 1995). Reversely, ventral most domains do not form in *Shh*<sup>-/-</sup> mutants (Figure 5) (Chiang et al., 1996). However, most functional studies of Shh morphogen are based on cellular protein levels and very little is known about the direct transcriptional mechanisms controlling target gene expression. In Paper I, we address this question and look at direct transcriptional events through which the extracellular Shh gradient regulates the intracellular activation of HD and bHLH genes.

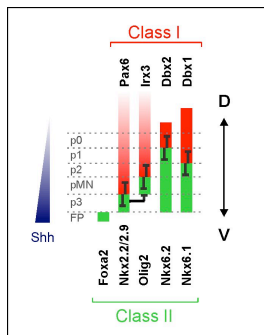


Figure 3. Cross-repressive interactions between complementary pairs of class II genes (activated by Shh) and class I genes (repressed by Shh) delineate sharp borders between adjacent progenitor domains in the ventral neural tube.

### **1.3. Shh pathway**

The first identified hedgehog (Hh) homolog was the *Drosophila Hh*, which was named for the appearance of pointy denticles in *Hh*<sup>-/-</sup> mutants, bearing resemblance to a hedgehog (Nusslein-Volhard and Wieschaus, 1980). The *Drosophila* genome was found to contain only one *Hh* gene, whereas three homologs were identified in the vertebrates: Sonic hedgehog (*Shh*), Desert hedgehog (*Dhh*) and Indian hedgehog (*Ihh*), all of which signal through the same pathway components (Echelard et al., 1993; van den Brink, 2007). Shh is the best-studied ligand of the hedgehog signaling pathway and the one that has been found to have the most critical roles in development. Shh is secreted by several organizing centers including the FP and notochord that pattern the

neuroectoderm as well as surrounding mesoderm and endoderm, and by the zone of polarizing activity (ZPA) that patterns the developing limbs. Moreover, Shh signaling affects other aspects of organogenesis, such as proliferation, apoptosis and axonal guidance (Bourikas et al., 2005; Cayuso et al., 2006). Conversely, Dhh is expressed in the developing testis and Ihh by visceral endoderm and both are dispensable for the CNS development (van den Brink, 2007).

The hedgehog pathway is largely evolutionarily conserved; nevertheless there are prominent differences between vertebrate and *Drosophila* hedgehog signaling components. In the following sections, I will primarily discuss the vertebrate system unless otherwise stated.

### **1.3.1. Shh protein**

Shh molecules are first detected at the ventral midline at the onset of patterning (mouse embryonic day 7.25), whereupon the quantity of Shh progressively increases and diffuses dorsally accumulating at the apical pole of the neural progenitors (Chamberlain et al., 2008). Shh is initially produced by the notochord, which remains in close contact with the overlying neuroectoderm for a short time following neural tube closure and serves as the only supply of Shh. In amniotes, notochordal Shh induces a second source of Shh production at the ventral midline of the developing neural tube, i.e. the presumptive FP. At spinal cord levels the notochord regresses ventrally, away from the neural tube, subsequently leaving the FP as its main source of Shh for the remaining period of the embryogenesis (Figure 1A) (Marti et al., 1995; Roeling et al., 1995; Roelink et al., 1995).

Shh is produced as a large precursor protein that prior to secretion undergoes a series of post-translational modifications to produce a biologically active molecule. The precursor protein is autocatalytically cleaved to produce an N-terminal fragment, which is further palmitoylated at the N-terminus by skinny hedgehog (Skn) and modified with a cholesterol moiety at the C-terminus (Dessaud et al., 2008; Matisse and Wang, 2011; van den Brink, 2007). Both modifications are essential for the activity of the secreted protein and play critical roles in determining range and shape of the extracellular gradient of Shh (Guerrero and Chiang, 2007; Huang et al., 2007).

The multi-pass transmembrane protein Dispatched1 (Disp1) regulates the release of active Shh molecules at the cell surface (Ma et al., 2002). The precise conformation in which Shh is secreted and how it diffuses away from the source and through the target field remains an open question. The leading hypothesis is that Shh monomers assemble into micelle-like, multimeric structures, thereby neutralizing the inherent hydrophobicity and changing diffusion properties. The post-translational lipid modifications of Shh are essential for assembly into these multimeric, high molecular weight complexes. In *Skn*<sup>-/-</sup> mutants, Shh is not palmitoylated and is released a monomer (Chen et al., 2004; Dessaud et al., 2008; Matisse and Wang, 2011), resulting in detection of Shh only in the ventral midline (Chamberlain et al., 2008). By contrast, artificially produced Shh that lacks the cholesterol moiety can be released independently of Disp1 and has a greater extracellular diffusion rate (Huang et al.,



2007). After the secretion, Shh molecules diffuse through the extracellular space away from the source and expose the target cells to different concentrations of the morphogen.

### 1.3.2. Intracellular transduction of Shh signaling

Once Shh has reached the responding cells, how do the cells transduce Shh-signal into a precise gene expression output? Despite the significant gaps in our knowledge, an outline of the mechanisms involved in intracellular Shh signal transduction is beginning to emerge.

Across the cell membrane

The activation of the hedgehog pathway in target cells is initiated by binding of Shh to the twelve-pass transmembrane receptor Patched1 (Ptc1). The binding of Shh induces inactivation and internalization of Ptc1, which leads to activation of the seven-pass transmembrane receptor Smoothened (Smo); the primary transducer of the intracellular Shh signaling cascade. Consequently, activated Smo initiates downstream intracellular events (Figure 4B). Conversely, in the absence of Shh binding, Ptc1 inhibits the activity of Smo and hence the hedgehog pathway is kept silent (Figure 4A).

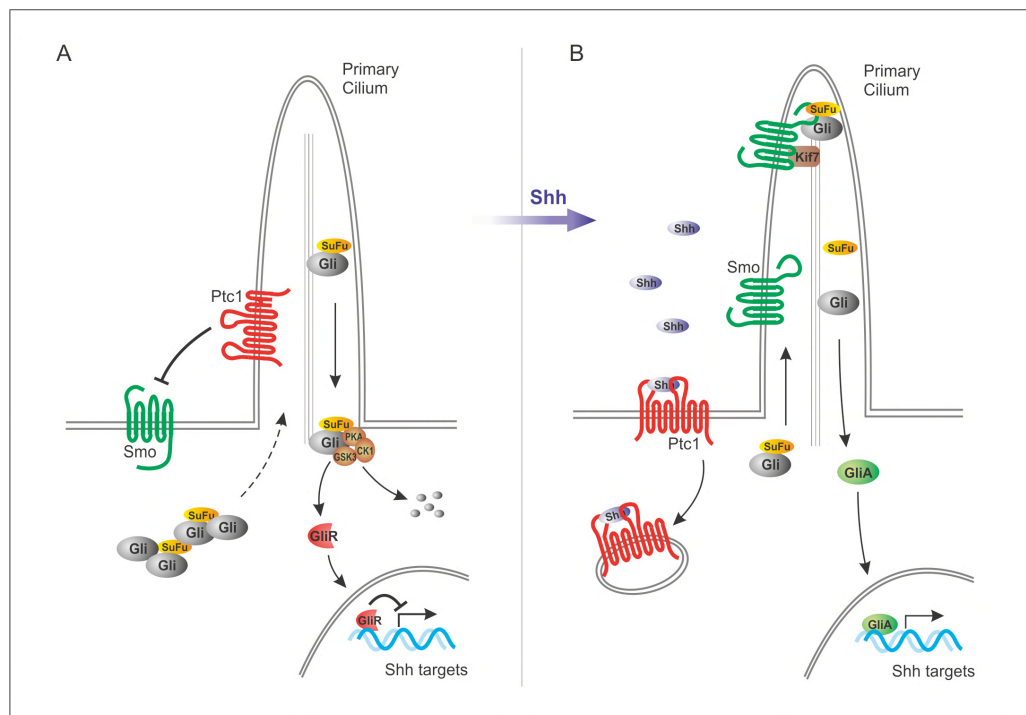


Figure 4. Schematics illustrating A) inactive and B) activated Shh signaling pathway.

A) In the absence of Shh, Ptc1 localizes to the cilium and inhibits the activity of Smo. Under these conditions, SuFu tatters full length Gli proteins in the cytoplasm and promotes Gli phosphorylation by PKA, CK1a and GSK3. Subsequently, the phosphorylated Gli are either completely degraded or truncated into repressor form (GliR) that translocates to the nucleus and inhibits the transcription of target genes. B) Binding of Shh to Ptc1 releases inhibition of Smo and causes internalization of Ptc1. Upon activation, Smo as well as SuFu-Gli and Kif7 accumulate in the tip of the cilia causing the disassembly of SuFu-Gli interaction. Full-length Gli proteins can thus become stabilized as activators (GliA), enter the nucleus and activate the target genes. (Adapted from JM Dias)

In *Drosophila*, Hh binding promotes endocytosis of Ptc and cell-surface accumulation of Smo (Denef et al., 2000). In vertebrates, this function is localized to the primary cilium, a microtubule-based narrow protrusion of the cell membrane. Primary cilia are essential for Shh signal transduction and all CNS progenitor cells extend an apical cilium into the ventricles of the neural tube. Importantly, Ptc1 is localized in the cilia in the absence of Shh, whereas ligand binding induces clearance of Ptc1 and active trafficking and accumulation of Smo into the ciliar tip (Figure 4A and 4B) (Corbit et al., 2005; Rohatgi et al., 2007).

Two homologs of *Drosophila* Ptc are expressed in overlapping patterns in the vertebrate CNS, *Ptc1* and *Ptc2*. However, genetic studies of *Ptc* mutants indicate that *Ptc1* has the essential role in Shh signaling during CNS development. *Ptc1*<sup>-/-</sup> mutant mice exhibit complete ventralization of the neural tube (Figure 5), whereas *Ptc2* is dispensable for normal patterning (Lee et al., 2006b). Interestingly, Ptc1 does not interact directly with Smo but rather functions indirectly to maintain it in a signaling-inactive state. Analyses of transmembrane domain of Ptc1 raises a possibility that Ptc1 regulates Smo by moving small regulatory molecules in and out of the cell; however, this has not been proven yet (van den Brink, 2007). How is the activation of Smo then achieved?

In *Drosophila*, upon pathway activation, Smo becomes phosphorylated in its C-terminal tail by Protein Kinase-A (PKA), Casein Kinase 1 alpha (CK1α) and Glycogen Synthase Kinase 2 (GSK2), a modification that leads to conformational change and activation of Smo. The vertebrate Smo sequence has deviated significantly from *Drosophila* in the C-terminal tail and lacks PKA phosphorylation sites. Nevertheless, vertebrate Smo can be phosphorylated by CK1α and GSK2. Shh promotes Smo phosphorylation by regulating its accessibility to CK1α/GRK2, resulting in a conformational change that promotes its ciliary accumulation (Chen et al., 2011) and subsequent activation of the intracellular Shh pathway.

*Smo*<sup>-/-</sup> mutants lack all domains ventral to p1 (Figure 5) (Wijgerde et al., 2002; Zhang et al., 2001) and in neural cell cultures most ventral cell fates can be induced by addition of small-molecule antagonists and/or antagonists of Smo (Chen et al., 2002; Zhou et al., 2010). Thus, the graded activation of Smo can recapitulate the cellular responses to graded Shh signaling.

#### Terminal effectors of Shh signaling

The Shh pathway converges on the Gli family of TFs: Gli1, Gli2 and Gli3 (homologues to the *Drosophila* Ci), which act as terminal effectors by regulating Shh target genes on the transcriptional level (Bai et al., 2004). Gli2 and Gli3 (and Ci) are bifunctional transcriptional repressors and activators (Ruiz i Altaba, 1999), which in the presence of Shh signaling are stabilized as activators, whereas in the absence of Shh signaling they are processed into transcriptional repressors.

In the absence of Shh signaling, Gli2 and Gli3 (and Ci) are constitutively phosphorylated by PKA, CK1 and GSK3 kinases at conserved sites located in the C-terminal half of each protein (Figure 4A). Hyperphosphorylated Gli2 and Gli3 (and Ci)

are thereafter targeted to proteasomes, where Gli3 (and Ci) proteins undergo proteolytic cleavage of the C-terminus, generating a truncated protein with repressor functions (Figure 4A) (Tempe et al., 2006). Similarly, Gli2 is also catalytically processed, but the majority of the protein is fully degraded and only small fraction of Gli2-repressor is formed (Pan et al., 2006). Activation of the Shh pathway inhibits Gli2 and Gli3 (and Ci) phosphorylation and stabilizes their full-length, activator forms (Figure 4B). On the other hand, Gli1 is a direct target of Shh signaling and it does not appear to have repressor activity. Additionally, Shh also regulates the nuclear accumulation of GliA, which are transported out of the nucleus in the absence of Shh signaling.

Consequently, the extracellular Shh concentration is converted to an intracellular ratio of Gli activators (GliA) and Gli repressors (GliR), the ratio of which represents the level of Gli activity within the cell. Importantly, alterations of intracellular Gli activity are sufficient to cell-autonomously mediate full range of Shh responses in the neural tube, positioning Gli activity as intracellular correlate of the Shh gradient (Stamatakis et al., 2005).

#### From Smo to Gli

While the cascade of events by which Smo regulates Gli-activity is not completely resolved yet, studies in *Drosophila* have elucidated certain mechanisms involved in signal transduction. In the absence of Hh, the kinesin-related scaffold protein Costal2 (Cos2) tethers Ci in the cytoplasm. Cos2 interacts with the kinases Fused (Fu) and Suppressor of fused (SuFu) in a complex that allows efficient phosphorylation of Ci and hence the formation of Ci-repressors (Matisse and Wang, 2011). Upon pathway activation, a conformational change in the C-terminal tail of Smo allows Smo to physically bind Cos2. Cos2 is thus recruited to the plasma membrane leading to release of Ci and stabilization of Ci-activators.

In vertebrates, there are two identified Cos2 orthologs; Kif7 and Kif27, of which Kif7 plays a conserved negative role in Shh pathway. Accordingly, Kif7 interacts with Smo and all three Gli proteins in vitro and the Kif7 null mice exhibit phenotypes associated with compromised GliR function (Cheung et al., 2009; Liem et al., 2009). However, neither in vivo interaction between Smo and Kif7 nor the direct tethering of Gli proteins by Kif7 has yet been shown. Furthermore, the vertebrate Fu is dispensable for neural tube patterning, whereas SuFu has several eminent functions. SuFu binds directly to Gli2 and Gli3 full-length proteins, anchoring them to the cytoplasm and hence preventing their translocation and activity in the nucleus and in addition antagonizes proteosomal degradation of Gli proteins. Thereby, SuFu regulates the amounts of full-length Gli in the cell (Figure 4A). Moreover, SuFu promotes phosphorylation of Gli2 and Gli3 to promote GliR formation. Accordingly, inactivation of SuFu in mice leads to ectopic pathway activation (Cooper et al., 2005). However, due to increased proteosomal degradation, the maximum activation of the Shh pathway cannot be achieved in *SuFu*<sup>-/-</sup> cells (Chen et al., 2011). Moreover, following pathway activation, SuFu is required for trafficking of Gli2 and Gli3 proteins into the tip of cilium. Concurrently, Smo and Kif7 are also trafficked into the cilium, an accumulation

that may promote the disassembly of SuFu-Gli interaction thus allowing full-length Gli to become stabilized as activators (Figure 4B).

Most Shh pathway components are dynamically trafficked in and out of the primary cilium, emphasizing the importance of cellular compartmentalization for mammalian signal transduction. Many studies have therefore focused on identifying components involved in cilia formation and ciliar trafficking. Several intraflagellar transport proteins (IFT) that build up ciliar structures, e.g. Ift172, Ift88 (Huangfu and Anderson, 2005), Arl13 (Larkins et al., 2011) and IFT25 (Keady et al., 2012), in addition to IFTs that control anterograde and retrograde motors, e.g. Dnchc2 and Kif3a (Huangfu and Anderson, 2005) and associated ciliar proteins, e.g. Ecv2 (Dorn et al., 2012), have been shown to be essential for Shh signal transduction elucidating several missing links between Smo and its mediatory Gli TFs.

### **1.3.3. Gradient interpretation: the battle between activators and repressors**

Shh signals through Gli TFs however, apart from direct activation of *Nkx2.2* and *FoxA2* expression (Lei et al., 2006; Sasaki et al., 1997) it has not yet been resolved whether class I and class II genes are directly regulated by Gli proteins. Moreover, Shh activity stabilizes GliA and prevents the formation of GliR, raising the question of whether Gli-mediated patterning mechanisms involve activation, repression or a combination of the two.

Analysis of mouse mutants with altered Shh/Gli activities indicates that class I and II genes differ in their requirements of Gli. In mutants such as *Shh*<sup>-/-</sup> and *Smo*<sup>-/-</sup>, in which Shh signaling and therefore all GliA has been ablated, class II genes that specify the ventral domains (FP, p3, pMN and p2) are absent, demonstrating a requirement of Shh-mediated activation (Figure 5). Conversely, reducing GliR by genetic elimination of *Gli3* results in a dorsal expansion of intermediate progenitor domains (p0-p1), whereas the generation of the remaining ventral cell types is unaffected (Figure 5) (Persson et al., 2002). However, partial redundancy with Gli2 mediated repression may account for this mild phenotype. Importantly, over-expression of a truncated form of Gli3 that acts as constitutive repressor blocks Shh responses throughout the ventral neural tube. Therefore a model has been proposed in which the GliA gradient is directly interpreted in the ventral neural tube, with GliR acting primarily to repress genes at dorsal positions (Persson et al., 2002). Furthermore, reduction of GliR in mutants in which Shh signaling has been eliminated, i.e. *Shh*;*Gli3* and *Smo*;*Gli3* double mutants, results in the restoration of several ventral cell identities (pMN, p2, p1 and p0) indicating that their generation can occur in the absence of GliA, as long as the repressive activity of Gli3 is removed (Figure 5). Therefore it has been alternatively suggested that the ratio between GliA and GliR regulates patterned gene expression (Bai et al., 2004; Ingham and Placzek, 2006).

Remarkably, neither FP nor p3 cells are generated in *Shh*;*Gli3* and *Smo*;*Gli3* double mutants, arguing that the induction of the ventralmost cell identities differs in Gli requirements. Moreover, similar phenotypes are observed in the mutants in which GliA

levels have been reduced. In *Gli2*<sup>-/-</sup> mutants, no FP and only few p3 cells develop and while *Gli1*<sup>-/-</sup> mutants have no discernable spinal cord phenotype, in *Gli1*;*Gli2* double mutants the severity of the *Gli2*<sup>-/-</sup> phenotype is augmented (Figure 5) (Park et al., 2000). These observations imply that the generation of FP and p3 cell fates requires high levels of GliA in addition to removal of GliR. Notably, in these mutants other ventral cell progenitors are present in their normal D/V positions apart from pMN cells that expand ventrally across the midline, indicating a differential requirement of GliA in the generation of these more dorsal cell types compared to the ventralmost progenitors.

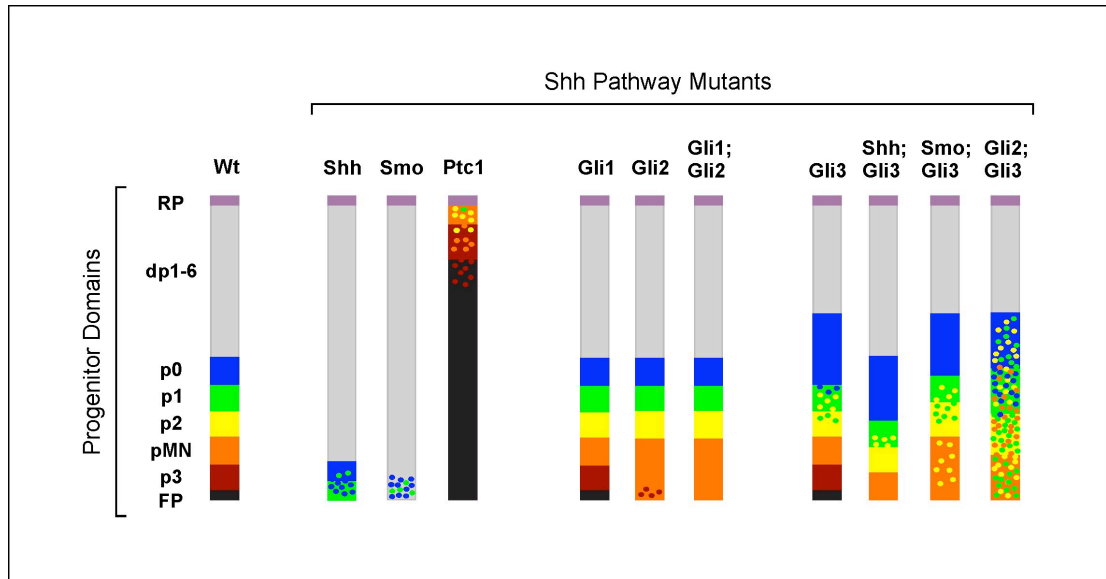


Figure 5. Schematics illustrating the changes in phenotypes within the ventral neural tube in mouse mutants where different components of the Shh pathway are inactivated, for details see the text.

Strikingly, in *Gli2*;*Gli3* double mutants that lack any form of Gli TFs, the p0-pMN progenitors are generated but develop as intermingled populations (Figure 5) (Bai et al., 2004; Lei et al., 2004), indicating that without input from Shh signaling the cells completely lack positional information in the ventral neural tube. Importantly, these observations also imply that additional signals can induce the generation of ventral domains independently of Shh signaling.

Even though these studies elucidate cellular mechanisms of target gene regulation, they provide little evidence of direct transcriptional regulation by Gli TFs. The partial redundancy between Gli3 and Gli2, together with the bi-functional nature of these proteins makes these mechanisms difficult to resolve. Paper I addresses these questions and proposes a novel mechanism of Shh-imparted positional information in which transcriptional regulation by GliA and GliR variants is distinguished.

#### 1.3.4. Shaping the gradient

A number of membrane proteins and extracellular matrix components that bind Shh protein have been identified. They play important roles in modulating the extracellular

Shh gradient as well as the intracellular signal output. These proteins can be grouped into those that promote or amplify Shh signaling and those that oppose or antagonize it.

#### Signal enforcement

Shh binding transmembrane receptors, such as Cdo, Boc and Gas1, enhance the signal in a cell-autonomous manner and are also down-regulated by the signal itself. Whether they augment the signal by increasing the ligand concentration or by a more active role in the signal transduction remains to be determined (Ribes and Briscoe, 2009). Analysis of *Cdo* and/or *Gas1* mutants shows that they are required for the generation of ventral cell fates. Lowering gene dosage of Cdo and Gas1 progressively increases the severity of the phenotype, to a point where none of the FP, p3 nor pMN domains are formed in the *Gas1;Cdo* double mutants (Allen et al., 2007).

#### Signal antagonism

Negative regulators of Shh signaling either restrict Shh diffusion or increase Shh degradation. In addition, some negative regulators are themselves targets of Shh signaling, creating auto-regulatory loops that influence the response of the receiving cells. The transmembrane receptor, Hedgehog Interacting Protein (Hhip), competes with the primary Shh receptor Ptc1 for binding of Shh, thereby lowering the availability of the ligand. Moreover, Ptc1 is internalized upon Shh binding, thus sequestering the ligand from further spread and targeting it for degradation. Additionally, extracellular matrix components such as megalin receptors and heparin sulphate proteoglycans, have been shown to bind Shh and restrict its diffusion (Guerrero and Chiang, 2007; McCarthy et al., 2002). As ligand availability and diffusion are decreased, Shh signaling is attenuated at distant positions in the field, reshaping the extracellular Shh gradient. Remarkably, both Ptc1 and Hhip expression is positively regulated by Shh, leading to the cell-autonomous in addition to non-autonomous inhibition of signal transduction. Conversely, complete elimination of Ptc1 expression results in de-repression of Shh signaling in the entire neural tube (Figure 5) (Motoyama et al., 2003). Moreover, in embryos lacking one or both *Ptc1* and *Hhip* alleles, cells belonging to adjacent progenitor domains are extensively intermixed instead of sharply delineated, showing that negative feedback likely contributes to buffer and stabilize potential fluctuations in the ligand concentration (Jeong and McMahon, 2005).

#### Temporal adaptation

During neurulation period, spinal cord progenitors exhibit a 'dorsal' character, expressing Pax3/6/7 genes, which are successively displaced to more dorsal positions by induction of progressively more ventral genes. At ventral midline, *Nkx6.1* is induced first and then *Olig2*, which is ultimately displaced by *Nkx2.2*. During the time of ventral progenitor induction, the amplitude of Shh gradually increases (Chamberlain et al., 2008) and consequently, the ventral progenitors are exposed to higher Shh signaling for longer period than adjacent dorsal progenitors. These observations imply that in addition to concentration, the duration of Shh signaling might also influence the cellular response. Correspondingly, progressively more ventral genes were activated in chick neural tube explant cells when the exposure time to Shh was prolonged, at the

expense of more dorsal genes (Dessaud et al., 2010; Dessaud et al., 2007). Thereby the induction of *Dbx1*, *Nkx6.1*, *Olig2* and *Nkx2.2* was proposed to be dependent on both concentration and duration of Shh reception. Notably, the abrogation of Shh signaling in cells exposed beforehand to high Shh concentrations led to conversion of the cell-fates to more dorsal identities, indicating inherent plasticity within the progenitor pool (Dessaud et al., 2010).

The gradual increase of *Ptc1* and *Hhip* expression in progenitor cells exposed to increasing concentration of Shh, indicates a progressive cellular adaptation to the signaling strength. Interestingly, the adaptation mechanism has also been proposed for the intracellular Gli activity, which appeared to be disproportional to the extracellular Shh concentration. By measuring Gli-mediated activation of luciferase reporter in chick neural tube explants treated with increasing concentrations of Shh, the authors observed that the maximal output of Gli activity is achieved at 1nM Shh, a concentration far below that required for induction of *Nkx2.2* in the same cells and the same time span (6h) (Dessaud et al., 2010; Dessaud et al., 2007). Interestingly, the Gli activity gradually decreased over a 24h period and this decrease was smaller at higher Shh concentrations, implying that higher concentrations of Shh are required to sustain the level of Gli activity necessary for induction of the ventral cell types (Dessaud et al., 2007).

Collectively, these observations suggest that the level of Shh pathway activation in progenitor cells is affected by negative and positive inputs that adjust both the extracellular concentration of Shh molecules and the intracellular Gli activity. Therefore, the dynamic adaption mechanisms play an important role in patterning output. Paper II addresses these questions and unravels a novel mechanism of cell-intrinsic modulations of Shh signaling in which Shh-concentration-independent step-regulation of Gli activity is discussed.

## **1.4. Positional information imposed on cells by dorsal and lateral signals**

In *Gli2;Gli3* double mutants, in which all Gli input is absent, positional information is lost in the ventral half of the neural tube (Figure 5). However, in these mutants the dorsal progenitor domains are established at appropriate positions, indicating that morphogens from dorsal and lateral sources also provide progenitors with important positional information.

### **1.4.1. BMP signaling**

Bone Morphogenic Proteins (BMP) belong to the Transforming Growth Factor beta (TGF $\beta$ ) superfamily of extracellular ligands and act at multiple stages of neural development, starting from the early requirement of BMP inhibition for the induction of neuroectoderm. As development proceeds, BMP production becomes localized predominantly to the dorsal structures of the CNS, such as the RP of the spinal cord. BMP2, BMP4 and BMP7 are secreted by the chick RP and BMP4, BMP6 and BMP7

by the mouse RP, whereas BMP inhibitors (noggin, chordin and follistatin) are secreted by the notochord and/or paraxial mesoderm (Liu and Niswander, 2005). Thereby a high-dorsal to low-ventral gradient of BMP activity is established in the developing spinal cord.

#### BMP pathway

BMP signaling is initiated by binding of BMPs to the transmembrane receptors belonging to the serine-threonine kinase family, namely BMP-type II-receptors (BMPRII) and BMP-type I-receptors, which include Alk2, Alk3 (also known as BMPRIa) and Alk6 (also known as BMPRIb). Ligand binding facilitates and stabilizes heteromeric complex formation between BMPRII and BMPRI and leads to efficient transphosphorylation of BMPRI by the constitutively active BMPRII subunit. Activated BMPRI subsequently phosphorylates receptor-regulated Smad TFs (R-Smads; Smad1, Smad5 and Smad8), the intracellular effectors of BMP signaling (Blitz and Cho, 2009; Liu and Niswander, 2005). Other R-Smads; Smad2 and Smad3, are regulated by different TGF $\beta$  superfamily members. Phosphorylation of R-Smads induces a conformational change freeing interaction between the DNA-interacting domain (Mad Homology 1, MH1) and the protein interacting domain (MH2). Phosphorylated R-Smads can then form complexes with co-Smad, Smad4, and accumulate in the nucleus where they bind to specific DNA motifs and activate target genes (Blitz and Cho, 2009; Liu and Niswander, 2005).

BMP inhibitors bind directly to BMP ligands in the extracellular space, blocking the interaction between the ligand and its receptors. In addition, BMP signaling can be modulated by inhibitory Smads (Smad 6 and Smad7), which compete with R-Smads at BMPRI phosphorylation sites, thereby preventing R-Smad binding to co-Smads and further downstream signaling (Blitz and Cho, 2009; Liu and Niswander, 2005).

#### Patterning by BMP

BMP signaling positively regulates proliferation and progenitor specification of dorsal neural subtypes in a concentration-dependent manner. In the chick neural tube, constitutive activation of BMP signaling induces ectopic expression of dp1 progenitors and DI1 neurons at the expense of the more ventrally located dp2 and dp3 domains (Timmer et al., 2002). Conversely, weak activation of BMP signaling causes ventral expansion of dp2 domain and DI2 cell fate at the expense of dp3 and DI3 interneurons (Timmer et al., 2002). Likewise, the spinal cord of mouse embryos that lack both BMPRIa and BMPRIb exhibit complete loss of dp1 and DI1 cells and a substantial reduction of dp2 and DI2 cells (Wine-Lee et al. 2004). In addition, ectopic ventral expression of BMPs induces the dorsal patterning genes *Msx1*, *Msx2* and *Pax7*, and represses the intermediate patterning genes *Dbx1* and *Dbx2* (Timmer et al., 2002). Moreover, in the case of *Msx1* and *Msx2*, activation has been proposed to be mediated directly by Smad1 (Alvarez Martinez et al., 2002; Brugger et al., 2004). Accordingly, these studies argue that discrete levels of BMP signaling are required correct patterning of the dorsal spinal cord.



In contrast, suppression of BMP signaling is crucial for normal patterning of the ventral spinal cord. Addition of BMPs alters the response of chick intermediate neural tube explants to Shh, such that cells adopt more dorsal phenotypes and do not induce the expression of the direct Shh targets *Ptc1* and *Foxa2* even at high Shh concentrations. Conversely, the BMP antagonist follistatin augments the response to Shh so that the same explant cells adopt more ventral fates (Liem et al., 2000) and ectopic expression of BMP antagonist chordin in the chick spinal cord expands the FP (*FoxA2*<sup>+</sup> cells) domain dorsally (Patten and Placzek, 2002). These observations indicate an interplay between the BMP and Shh signaling pathways during neural pattern formation.

#### **1.4.2. RA signaling**

Retinoic Acid (RA) is a small lipophilic molecule expressed by paraxial mesoderm and later by somites, positioning RA in relation to the neural tube as a laterally diffusing signal. RA is synthesized from vitamin A in two sequential steps of oxidization, involving cytosolic alcohol dehydrogenases (ADH) or membrane-bound retinol dehydrogenases (RDH) and subsequently retinaldehydes (RALDH), of which RALDH2 is expressed at high levels by paraxial mesoderm and somites (Maden, 2006). Additionally, enzymes of the cytochrome P450 26 subfamily; CYP26A1, CYP26B1 and CYP26C1, act as negative regulators of the RA gradient by catalyzing reactions that metabolize RA (Rhinn and Dolle, 2012). Both excess and deficiency of RA can cause neuronal malformations, implying that the spatial and temporal distribution of RA are critically important (McCaffery et al., 2003).

##### RA pathway

The extracellular RA molecule diffuses through the cell and nuclear membranes into the nucleus where it binds to retinoic acid receptors (RAR) that belong to the nuclear receptor superfamily (Rhinn and Dolle, 2012). Three identified RARs, RAR $\alpha$ , RAR $\beta$ , and RAR $\gamma$ , are conserved throughout vertebrate evolution and form heterodimeric complexes with retinoid X receptors, RXR $\alpha$ , RXR $\beta$ , and RXR $\gamma$ , a complexes that facilitates their binding to specific DNA motifs. Prior to exposure to RA, RAR/RXR complexes pre-bind the DNA and recruit co-repressors, whereas upon ligand binding, RARs undergo a conformational change that leads to release of co-repressors and recruitment of co-activators (Rhinn and Dolle, 2012). Thereby, RA signaling activates the target genes that have previously been actively suppressed by RAR/RXR. Notably, the recruitment of RA might also induce de novo binding of RAR/RXR complexes to previously unbound sites in genome (Mahony et al., 2011).

##### Patterning by RA

A possible morphogen activity for RA comes primarily from the hindbrain studies, in which increasing concentrations of RA are required for induction of progressively posterior hindbrain compartments. Importantly, cellular experiments have shown that RA activates genes expressed by the intermediate spinal cord; *Pax6*, *Irx3*, *Dbx1* and *Dbx2*. In addition, RA can induce expression of *Olig2* independently from Shh signaling (Maden, 2006; Novitsch et al., 2003; Pierani et al., 1999). Conversely,

inhibition of RA signaling by over-expression of a dominant-negative RAR in the chick spinal cord led to repression of *Pax6*, *Irx3*, *Dbx1*, *Dbx2* and *Olig2*. In parallel, dominant-negative RAR inhibits expression of post-mitotic genes associated with the V0, V1, V2 and MN fates even in the presence of their progenitor determinants (Novitsch et al., 2003), indicating that RA signaling is important for the acquisition of neuronal subtype properties in addition to activation of patterning progenitor genes.

### **1.4.3. Tissue-specificity**

Morphogens are pleiotropic signals with multiple functions during embryogenesis that pattern several tissues from the same source. In addition to patterning of neural plate derivatives including the neural tube and eye, Shh signaling has also been implicated in the early specification of the neural crest derivatives and somite lineages, patterning of limb buds and the development of foregut such as lungs and pancreas, among others (Bowers et al., 2012; Litington et al., 1998). BMP and RA signaling also pattern many of these tissues. RA, in addition to regulating the development of the CNS and eyes, plays a role in development of limbs and several foregut derivatives including lungs and pancreas, and controls the vertebrate segmentation clock (Rhinn and Dolle, 2012). BMPs have been implicated in development of most tissues including induction and patterning of the neural plate and neural crest derivatives, but also limb development, induction of epidermis, induction of skeletogenesis and inhibition of myogenesis, to name a few (Bond et al., 2012; Robert, 2007). In each of these developing tissues, the noted pleiotropic signals activate and repress different sets of target genes. How does Shh, BMP or RA activate one set of genes in the CNS and another for example in the limb bud? Do the responses depend on developmental history of the cells via mechanisms such as epigenetic regulation or are they dependent on tissue-specific co-factors? Paper III elucidates a mechanism that allows the pleiotropic signaling pathways to execute neural-specific tissue responses bringing our knowledge a step closer to understanding these fundamental mechanisms.

## **1.5. Neurogenesis**

Neuronal cells are specified in the progenitor domains located in the ventricular zone of the neural tube, where progenitors proliferate to the appropriate numbers required for embryonic growth. When the cells become destined to exit the cell-cycle, they migrate away from the ventricular zone toward lateral edges of the spinal cord, down-regulating the progenitor genes and initiating expression of genes controlling mature neuronal features and specialized subtypes properties. The transition from proliferation to differentiation is tightly controlled to ensure the acquisition of the correct mature cell type, which is enforced by the TF code in the progenitor at the time of cell-cycle exit. This maturation process is called neurogenesis. Proneural genes are the main driving force behind neurogenesis, whereas SoxB1 and Notch proteins counteract it (Kiefer, 2007; Zhou et al., 2010).

### **1.5.1. Proneural genes**

Proneural genes encode TFs of the bHLH family that in mouse include *Ngn1-3*, *Ascl1/Mash1* and *Math1/5*. These TFs are induced at high levels in progenitor cells after the patterning has been established. Proneural genes are able to induce neurogenesis by activating pan-neuronal genes, such as  *$\beta$ tubulin*, *NeuroD* and *Early B-cell factor 2 and 3*, which endow the differentiating cells with general neuronal properties. Simultaneously, proneuronal genes induce expression of the Notch ligands Delta and Jagged, which attenuate Notch signaling cell-autonomously while activating it in neighboring cells. Activated Notch signaling negatively controls both the expression and activity of proneural genes thereby cell-autonomously inhibiting differentiation (Bertrand et al., 2002). The balance between these factors therefore controls decision to remain a progenitor or to differentiate. In parallel, vertebrate proneural genes promote cell-cycle exit by inducing the expression of cyclin-dependent kinase inhibitors, although *Ngn1-3* promote cell-cycle exit with higher efficiency than the *Math* and *Mash* genes (Bertrand et al., 2002).

Interestingly, in certain lineages proneural genes are also cell fate-determining factors and are able to induce subtype specific TFs while simultaneously inhibiting gliogenesis through blockage of signals involved in glial differentiation (Bertrand et al., 2002). An example in the ventral spinal cord is the post-mitotic marker of somatic motor neurons, Hb9, which is induced by cooperative activities of Olig2 and Ngn2. Olig2 is also required for oligodendrocyte differentiation, which commences upon down-regulation of Ngn2 (Allan and Thor, 2003).

### **1.5.2. SoxB1 proteins**

Sox family members belong to the high mobility group (HMG) superfamily, characterized by an HMG domain responsible for sequence-specific binding to the DNA minor grooves, a binding that results in bending of DNA at an angle of 30-113° (Kamachi et al., 2000). There are several groups of Sox proteins designated based on phylogenetic analysis of their HMG domains and further subdivided by similarity of additional domains, and they play many functions in various tissues. SoxB1 subgroup members, Sox1, Sox2 and Sox3, share greater than 90% amino acid residue identity in their HMG domains and contain a transactivation domain. Sox1-3 are functionally redundant TFs expressed in the early embryo, the developing testis and CNS progenitors, where they control cell fate commitment (Miyagi et al., 2009; Pevny and Placzek, 2005). In neural tissue, Sox1-3 prevent differentiation by keeping cells in a proliferative state, in part by preventing proneural activities (Bylund et al., 2003; Graham et al., 2003; Miyagi et al., 2009), and they are generally down-regulated upon neural differentiation.

In contrast to the SoxB1 group, other Sox members, Sox21, Sox11 and Sox4, have been implicated in promoting neurogenesis. Expression of Sox21 (a SoxB group member containing a repressor domain) is upregulated by proneural factors in progenitor cells, and, upon reaching a threshold concentration Sox21 represses Sox1-3

activity to create an environment permissive for differentiation (Kiefer, 2007; Sandberg et al., 2005). On the other hand, Sox4 and Sox11 are expressed in early post-mitotic neurons, in which they positively regulate the expression of pan-neuronal markers and endow cells with general neuronal properties uncoupled from cell-cycle exit and differentiation (Kiefer, 2007).

## 2. AIMS

The research presented in this thesis aims to give better understanding of morphogen-mediated regulation of patterned gene expression that forms the basis for generation of specific neuronal subtypes in the developing CNS. More specific aims are:

- To investigate transcriptional mechanisms by which Shh gradient regulates spatial expression of cell fate-determining genes in the developing spinal cord.
- To study cell-intrinsic mechanisms involved in non-graded regulation of Shh signaling activity.
- To investigate transcriptional mechanisms by which Shh and other pleiotropic signaling pathways induce neural-specific responses.



### 3. RESULTS AND DISCUSSION

The mechanisms by which graded information provided by morphogens is interpreted at the genomic level and translated into precise expression patterns of target genes have not been resolved in vertebrates. The following discussion, based on Papers I, II and III presented in this thesis, focuses on Shh morphogen interpretation in patterning of the ventral neural tube. The discussion offers an explanation of gradient governed transcriptional mechanisms that impose positional information on progenitor cells (Paper I), it further revolves around non-graded intracellular modulation of the Shh-pathway strength (Paper II) and finally deliberates on neural tissue-specific responses to Shh and other pleiotropic pathways (Paper III).

#### 3.1. The *cis*-regulatory logic of gene regulation

The mechanisms by which morphogens convey the positional information are discernible in interactions between morphogen-transducing TFs and the regulatory regions of target genes. In view of that, we started the study in Paper I by identifying gene regulatory elements for patterning genes expressed by ventral neural tube.

The transcriptional activity of individual genes is largely determined by the *cis*-regulatory modules (CRM) located within the non-coding regions in the proximity of the coding sequences (Hardison and Taylor, 2012). Binding of specific co-activators to the CRMs recruits RNA polymerase II and the associated transcriptional machinery. Alternatively, the negative regulators bind CRMs and recruit repressor complexes. Recent advances in bioinformatics programing have made it possible to screen for CRMs by searching evolutionally conserved DNA sequences at varying distances from the transcriptional start site. The length of identified CRMs, ranging from a short sequence to a few hundreds base pairs (bp), makes these sequences simple to isolate and clone into reporter vectors. The CRM-driven reporter expression can then easily be tested for activity by various techniques including in vivo electroporation and in vitro transcriptional assays (for further descriptions see the experimental procedures of Papers I and III). Importantly, the clustering of conserved binding sites within the CRMs can indicate which signaling pathways and other transcriptional mechanisms converge on these elements.

##### 3.1.1. Isolation of CRMs regulating spinal cord TFs

Shh pathway converges on Gli TFs that all contain a highly conserved zinc-finger domain, which mediates binding to the specific DNA motifs in the genome (Kinzler and Vogelstein B, 1990). We have screened the conserved non-coding sequences surrounding genes regulated by Shh for the presence of conserved Gli-binding sites (GBS) and were able to isolate CRMs of the following neural genes: *Nkx2.2*, *Nkx2.9*, *Nkx6.1*, *Nkx6.2*, *Olig2*, *Dbx1*, *Dbx2* and *Pax6*. In Paper I we show that, when expressed

in the chick neural tube, these sequences recapitulate the expression patterns of their respective endogenous genes, indicating that each CRM has sufficient *cis*-regulatory information to control the corresponding gene expression.

We have isolated one functional element for each gene, however we cannot exclude the possibility of the existence of additional regulatory elements and we did not try to express these CRMs in other tissues where these genes are developmentally expressed. For example, the regulation of *Pax6* involves few independent enhancers that direct *Pax6* expression to distinct regions such as the eye, pancreas or neural tube (Zhang et al., 2003). Furthermore, due to the constraints of the methods used in these studies, we were only able to test the activity of the isolated CRMs from Hamburger–Hamilton chick developmental stage 13 (HH13) (6 hours post electroporation; hpe) until HH23 (40hpe). Therefore we cannot argue that these elements contain sufficient information for complete temporal regulation in the developing neural tube. Generation of stable transgenic animals either expressing reporter under the control of each CRM or with a complete deletion of these elements in the mouse genome are needed to address these issues.

### **3.2. Shh/Gli regulation of target genes by a direct transcriptional mechanism**

The isolated CRMs provide powerful tools to study the transcriptional mechanisms governing the expression of the patterning genes in the ventral CNS. Consequently, in Paper I we show that the ectopic activation of Shh signaling in neural tube achieved by electroporation of a constitutively active form of Smo (SmoM2) (Xie et al., 1998) activates the CRMs of class II proteins, whereas the CRMs of class I proteins are suppressed in the same manner as the endogenous genes they regulate. Moreover, the inactivation of GBSs by point mutations in CRM<sup>Nkx6.1</sup>, CRM<sup>Nkx2.2</sup>, CRM<sup>Nkx2.9</sup> and CRM<sup>Olig2</sup> abolished the activity of these CRMs, indicating a direct requirement of GliA binding for their activation. By contrast, the inactivation of the GBSs in CRM<sup>Nkx6.2</sup> and CRM<sup>Dbx1</sup> activated these CRMs ectopically in the dorsal neural tube, suggesting that the direct binding of GliR is required to establish correct dorsal *Nkx6.2* and *Dbx1* expression borders. Collectively, these results argue that Shh regulates neural patterning genes by a direct transcriptional mechanism, mediated by GliA and GliR binding to the regulatory sequences of the target genes.

### **3.3. Gradient readout at the CRM level: Gli activation or Gli repression?**

The partial redundancy between Gli proteins and their bi-functional nature renders the mechanism imposed by graded Shh signaling hard to resolve. In Paper I, we have constructed a set of experiments where we addressed this question from a direct transcriptional objective.



### **3.3.1. Positioning by the GliR gradient**

Every isolated CRM contains one or several functional GBSs consisting of a conserved consensus DNA motif that differs with one or more bp between the individual GBS in each CRM. Each nucleotide in the GBS motif influences Gli binding differentially, which can be estimated by positional weight matrix (PWM) (Hallikas and Taipale, 2006) that ultimately measures the affinity of the entire motif. Implementing this method to all GBSs in each CRM revealed that locally induced genes (*Nkx2.2*, *Nkx2.9* and *Foxa2*) contain a unique high quality GBS. In contrast, genes induced at distant positions are associated with variable numbers and generally lower quality of GBS. However, no correlation between the spatial position versus the number and quality of these GBSs could be predicted. Strikingly, the genes associated with the highest GBS affinities are located closest to the Shh source and thus the highest GliA levels, suggesting that if cells were merely reading the GliA gradient only the most ventral domains would be established. Remarkably, the GBS affinity appears inverse to the GliR gradient raising the possibility that dorsal repression by GliR is imposing positional information and regulating expression boundaries in a concentration dependent manner.

To explore if the GliR gradient is the instructive patterning mechanism, we altered the affinities of the essential GBS1 in the CRM<sup>Nkx6.1</sup> and the unique GBS in the CRM<sup>Nkx2.2</sup> by point mutations. Lowering the affinity of the indicated GBSs did not alter the expression of the CRM<sup>Nkx6.1</sup> nor CRM<sup>Nkx2.2</sup> in the endogenous ventral domain, however it lead to ectopic activity of these CRMs in the dorsal neural tube. These data strongly argue that GliR binding to qualitatively different GBSs sets the dorsal boundaries of the CRM activity. The data are also consistent with the ectopic dorsal activation of CRM<sup>Nkx6.2</sup> and CRM<sup>Dbx1</sup> carrying the inactivated GBSs. Moreover, lowering the levels of GliA and GliR in the spinal cord without effecting the GliA:GliR ratio, achieved by overexpression of Gli zinc-finger domain (GliZnf), shifted dorsally the expression domains of class I genes and the dorsal boundaries of class II genes. These observations suggest that GliR indeed is the restricting factor along the D/V axis and instructs spatial positioning of Shh-target genes at a transcriptional level.

Notably, lowering the levels of GliA and GliR by overexpression of GliZnf in the two most ventral domains reduced *Nkx2.2* expression as well as *Foxa2* (data not shown), concomitant with ventral expansion of *Olig2*. These data argue that the FP and p3 domains are differentially regulated by Shh and suggest that the extent of GliA binding through high-quality GBS is the determining mechanism for locally induced genes. This model is consistent with various Gli mutant studies where the loss of the two most ventral domains has been observed (see introduction), implying that induction of FP and p3 cells requires GliA to accumulate to a critical level necessary to counteract GliR-mediated repression.

The repression mechanism also provides a rationale for the temporal sequence of ventral gene induction in the developing spinal cord. The first response to Shh signaling is the decrease of GliR activity, allowing CRMs containing GBSs with sub-optimal binding to become rapidly induced. In contrast, genes associated with CRMs containing

high-affinity GBSs require Shh signaling to accumulate until appropriate threshold levels of GliA are reached and thus the onset of the expression for locally induced genes is prolonged.

### **3.3.2. Gene-activation mechanisms**

Ectopic activation of GBS-inactivated CRM<sup>Nkx6.1</sup> and CRM<sup>Nkx2.2</sup> in the dorsal progenitors, which have generally been presumed devoid of all GliA, implies that very low amounts of GliA are needed to activate these CRMs. Combined with the dorsal relocation of ventral domains in the GliZnf experiments, the data argues against a concentration dependent GliA mechanism for the transcriptional interpretation of long-range Shh signaling. Moreover, the data implies that additional transcriptional co-activators are facilitating Gli mediated activation.

A bioinformatics based cross-comparison of all isolated CRMs identified a general overrepresentation of SoxB1-binding sites (SBS) in each CRM suggesting that SoxB1 proteins could be general transcriptional co-regulators during neural patterning. Concomitantly, intact SBS are absolutely required for the activation of all isolated CRMs in vivo (Paper I and III). Moreover, GliA or SoxB1 alone are insufficient to induce in vitro activation of luciferase reporters regulated by the CRMs associated with class II genes (Paper I). Importantly, in combination, GliA and SoxB1, strongly activate these CRMs and require both intact GBSs and SBSs. Accordingly, the synergistic activities of SoxB1 and GliA provide a mechanistic rationale for a largely concentration independent mode of gene activation by GliA in neural progenitors.

Chromatin immunoprecipitation (ChIP) shows that Sox3 binds to all isolated CRMs (Paper I, Paper III and data not shown), irrespective of the activation state of their associated genes, raising the question whether SoxB1 also could facilitate GliR binding. In limb bud tissue where neither the neural Shh-target genes nor SoxB1 proteins are expressed, Gli3 repressors are bound to the neural CRMs (Vokes et al., 2008), indicating that GliR can stably bind without the input of SoxB1. However, we do not provide evidence that SoxB1 does not influence GliR binding properties and additional protein-protein-DNA assays are needed to resolve this issue.

Moreover, the ectopic activation of CRM<sup>Nkx6.1</sup> and CRM<sup>Nkx2.2</sup> in the dorsal progenitors indicates that Shh signaling occurs to some degree in the dorsal neural tube and that sufficient amounts of GliA are present there to trigger CRM activation. Our Western blot analysis showed that in addition to proteolyzed Gli3 repressor, also full length Gli3 protein is present in the dorsal progenitors (Paper I). Moreover, the signal enhancement in the domains exposed to the lowest Shh concentrations could be facilitated by Cdo and Boc receptors, which are expressed at high levels in the dorsal and intermediate parts of the neural tube (Mulieri et al., 2000; Tenzen et al., 2006).

### **3.3.3. Context dependence: long-range versus short-range activation**

CRM<sup>Nkx6.1</sup> and CRM<sup>Nkx2.2</sup> are activated in the correct ventral domain regardless if the essential GBS is mutated to resemble low affinity GBS<sup>Dbx1</sup>, GBS1<sup>Nkx6.1</sup> or high affinity

GBS<sup>Nkx2.2</sup> (Paper I). These observations indicate that GBS quality is subordinate to the context of additional binding motifs. However, the extent of context dependency varies between short- and long-range Shh targets.

To inactivate the long-range Shh target CRM<sup>Nkx6.1</sup> it is sufficient to mutate one SBS, the SBS2 abutting GBS1, indicating high dependency on the CRM architecture. Strikingly, the activity of the CRM<sup>Nkx6.1</sup> containing mutated SBS2 is restored when the affinity of the GBS1 is augmented (by converting GBS1<sup>Nkx6.1</sup> into GBS<sup>Nkx2.2</sup>) suggesting that higher GBS affinity renders gene activation less dependent on co-activators. In contrast, to inactivate the short-range Shh-target CRM<sup>Nkx2.2</sup> independent of GBS, three SBSs and two TCF/LEF binding sites (transcriptional mediators of Wnt signaling) (Lei et al., 2006) must be inactivated. Thus the activation of locally induced genes, due to association with high-quality GBS, appears to be less dependent on CRM context and cooperative input by co-activators.

Our data in Paper I define two separate mechanisms of Shh gradient interpretation at a transcriptional level; specification of the p3 domain compared to interpretation at long-range. However at the moment we cannot discriminate between mechanisms of spatial regulation for individual long-range Shh-targets. These genes are dorsally repositioned when GliR levels are reduced, however, we did not find any correlation between affinity and number of associated GBSs. Therefore, additional, yet to be discovered mechanisms implemented in the CRM architecture surrounding the GBSs are likely involved in GliR gradient interpretation when spatially positioning target genes at distance from the Shh source.

#### **3.3.4. Different tissue, same gradient**

It is interesting to consider if our novel mechanism of GliR regulated interpretation of the Shh gradient in the neural tube applies also to the other tissues patterned by Shh. In the developing limbs, Shh is secreted by the posterior source, i.e. ZPA, and patterns the A/P axis including the digit number and type. It has long been considered that the Gli3 repressor plays the major role in the development of the entire limb tissue as the *Gli1*;*Gli2* double mutants exhibit normal limb patterning (Park et al., 2000) whereas, the *Shh*;*Gli3* mutants exhibit similar phenotype to the *Gli3* single mutants (Litington et al., 2002; te Welscher et al., 2002). However, by temporally inactivating *Gli3* on the *Gli2*<sup>-/-</sup> background in combination with knockin of the *Gli1* into the *Gli2* locus, a recent study found that collective GliR levels are instructive in patterning of the anterior limb and the digit number, whereas the GliA are specifically required for the patterning of the most posterior autopod (Bowers et al., 2012). Thus the developing limbs appear to be patterned by a similar mechanistic logic; GliR levels are instructive at long-range from the ZPA, whereas the ratio between GliA:GliR levels is interpreted at close proximity to the ZPA.

### 3.4. HD repressive mechanisms

Genetic studies have shown that downstream of Shh signaling, cross-repressive interactions between pairs of patterning TFs are important to maintain sharp progenitor domain boundaries and reinforce the progenitor identities. However, if these repressive events involve direct transcriptional regulation remains elusive. We have further cross-compared the isolated CRMs in Paper I and observed an overrepresentation of conserved HD binding sites (HBS) in all identified CRMs. Characterization of these sites in CRM<sup>Nkx6.1</sup> revealed that two discrete HBSs are responsible for specific inhibition of the CRM<sup>Nkx6.1</sup> in the intermediate, Dbx1/2-expressing spinal cord and the dorsal spinal cord that expresses Msx1/2 in the chick and Msx1/2/3 in the mouse (Liu et al., 2004). We show that Msx1 and Dbx1 bind directly to the defined HBSs and that the Msx1/2/3 or Dbx1/2 site-specifically inhibit the CRM<sup>Nkx6.1</sup> activity in vitro. We extend this study in Paper I to show that Msx1/2/3 also repress endogenous *Dbx1* and *Dbx2* as well as CRM<sup>Dbx1</sup>. Interestingly, *Msx1/2* and *Nkx6.1* are not expressed in adjacent domains of the developing spinal cord, suggesting that many repressive partners that do not share expression boundary might yet be identified.

*Gsh1/2* encode HD-containing repressors involved in specification of dorsal neuronal subtypes (Kriks et al., 2005; Mizuguchi et al., 2006). However, very little is known about Gsh1/2 interactions with other patterning TFs. We overexpressed Gsh1 in the chick spinal cord and observed that Gsh1 is able to specifically repress *Dbx1* and *Dbx2* in vivo, as well as the CRM<sup>Dbx1</sup>, and this repression was accompanied by de-repression of *Nkx6.1* and CRM<sup>Nkx6.1</sup> in the Dbx domain (unpublished data presented in Figure 6A and data not shown). The addition of Gsh1 abrogated SoxB1-mediated activation of the CRM<sup>Dbx1</sup> in vitro, whereas it had no effect on SoxB1/GliA-mediated activation of the CRM<sup>Nkx6.1</sup> (Figure 6A), suggesting that the repression of *Dbx1* by Gsh1 is a direct transcriptional mechanism, (for description of electroporation and transcriptional assays see experimental procedures of Paper I or III). Similarly, Gsh2 and Dbx1 have been found to be cross-repressive partners in the *Xenopus* neural plate (Winterbottom et al., 2010), in which Gsh1 and Gsh2 were also able to repress *Irx3* expression (Winterbottom et al., 2011). These observations raise the possibility that the same Gsh1/2-mediated repressive mechanisms are occurring in the spinal cord of higher vertebrates and elevate the complexity of repressive interactions in the developing CNS.

Moreover, we have further investigated the effects of Msx1/2 proteins on other ventral patterning genes. We found that overexpression of Msx2 in the chick neural tube does not affect *Nkx6.2*, whereas it induces ectopic expression of *Nkx2.2* and *Olig2* (unpublished data presented in Figure 6B). However, SoxB1/GliA activation of CRM<sup>Nkx2.2</sup> and CRM<sup>Olig2</sup> in transcriptional assays was not affected by addition of Msx2 (Figure 6B), implying that the Msx2-mediated induction of *Nkx2.2* and *Olig2* is an indirect effect. The ectopic activation of *Nkx2.2* may be result, at least in part, of the observed repression of *Pax6* by Msx2 in these embryos (Figure 6B). However, Msx1/2 and Pax6 are co-expressed in the wild type neural tube implying that Msx2 regulates

expression of Pax6 and Nkx2.2 indirectly through a different set of transcriptional regulators.

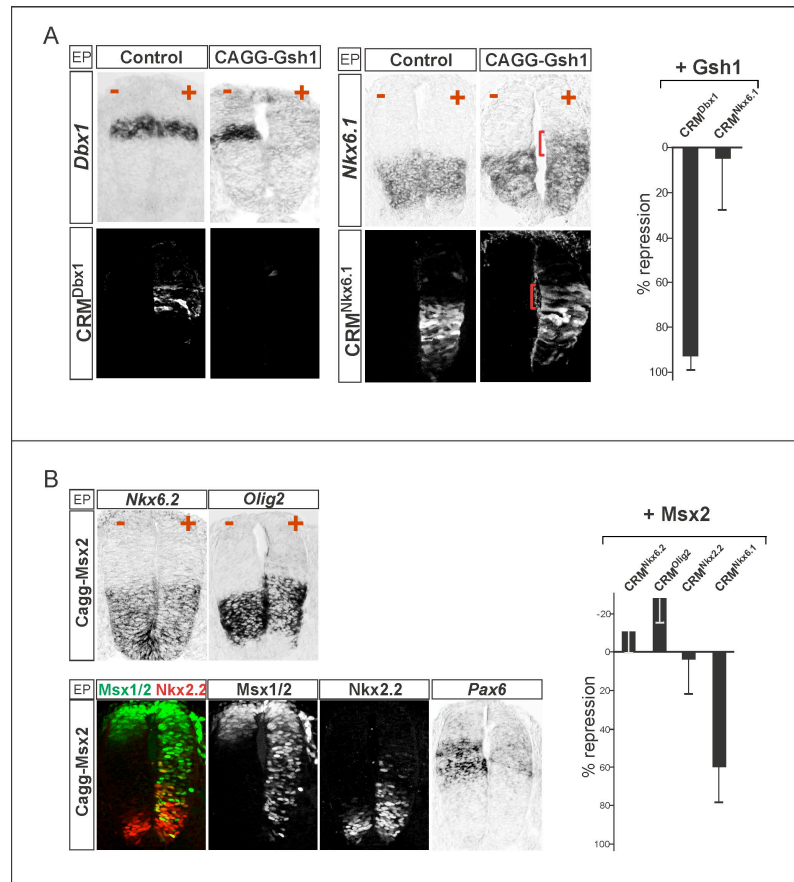


Figure 6. Repressive abilities of the dorsally expressed HD genes.

A) Overexpression of *Gsh1* in the chick neural tube represses *Dbx1* as well as the CRM<sup>Dbx1</sup>, resulting in dorsal expansion of the *Nkx6.1* domain as well as CRMNkx<sup>6.1</sup> activity. *Gsh1* exerts no direct effect on the *in vitro* activity of CRM<sup>Nkx6.1</sup> in P19 cells, whereas it acts as a potent repressor of CRM<sup>Dbx1</sup>. B) Overexpression of *Msx2* in the chick neural tube does not affect *Nkx6.2*, whereas the *Msx2*-mediated induction of *Olig2* and *Nkx2.2* is likely due to indirect repression of *Pax6* and possibly *Irx3*. *Msx2* exerts no direct effects on the *in vitro* activity of CRM<sup>Nkx6.2</sup>, CRM<sup>Olig2</sup> and CRM<sup>Nkx2.2</sup>. Luciferase activity of CRM in the presence of Sox2 for CRM<sup>Dbx1</sup> or Sox3 and GliA for remaining CRMs was set as a baseline. Percentage repression was calculated by comparing the baseline with the luciferase activity of the CRMs upon Sox2 or Sox3/GliA co-transfection with *Msx2* or *Gsh1*. Error bars indicate SD (n=2)

### 3.4.1. Repressive network: co-repression by GliR and HD

CRM<sup>Nkx6.1</sup> carrying the inactivated HBSs, characterized in Paper I, is activated in the dorsal neural tube where GliR levels are high. These observations imply that Gli-mediated repression is compromised when repression by HD proteins is impaired. Moreover, our experiments in Paper I also indicate that HD-mediated repression is impaired when GliR binding is weakened or absent. Lowering the GBS affinity in the CRM<sup>Nkx6.1</sup> and the CRM<sup>Nkx2.2</sup> causes their ectopic activation in domains that express HD TFs with ability to repress *Nkx6.1* and *Nkx2.2* genes: *Msx1/2* and *Pax6*, respectively. Similarly, the inactivation of GBSs in the CRM<sup>Dbx1</sup> and CRM<sup>Nkx6.2</sup> caused dorsal de-repression of these CRMs into progenitor domains that express

Msx1/2/3;Gsh1/2 and Dbx1/2, respectively, consistent with the data indicating that cross-repression between Dbx1 and Nkx6.2 in Gli3 mutants is impaired (Persson et al., 2002). Accordingly, we show that Msx1 and GliR coordinately repress CRM<sup>Nkx6.1</sup> transcriptional activity in vitro cell assays and propose that integrated HD and GliR repression is likely to apply to other Shh-regulated CRMs.

Moreover, the uniform activation of HBS inactivated CRM<sup>Nkx6.1</sup> throughout the D/V axis of the neural tube is partially de-repressed in early embryos (8hpe). These results suggest an overlapping temporal relationship between the GliR gradient and more region-specific repressive input necessary to robustly suppress ectopic gene activation in the neural tube.

In addition, in Paper III (discussed later in this section), we show that cross-repressive interactions between class I and class II TFs are recapitulated in the developing limb bud tissue when this set of genes is ectopically induced there, suggesting a mechanism utilizing common repressive complexes that are present in various tissues. Groucho co-repressors are known to interact with the terminal TFs of several signaling pathways (Cinnamon and Paroush, 2008) and are broadly expressed in many tissues including the neural tube and limb buds (Muhr et al., 2001; Van Hateren et al., 2005). Moreover, many neural HD repressors require direct binding to Groucho for their activity (Muhr et al., 2001). By contrast, Ci proteins were not found to interact directly with Groucho in *Drosophila* wing (Apidianakis et al., 2001) hence it is unlikely that GliR recruits Groucho in higher vertebrates either. However, when the ability of Groucho to interact with HD repressors is disrupted, the *Dbx2*, *Nkx6.1* and *Nkx2.2* are de-repressed in the dorsal spinal cord despite that GliR levels should be largely unaltered in these experiments (Muhr et al., 2001). Thus HD-Groucho interactions provide region-specific co-repressor complexes that are necessary to augment GliR responses. Likewise, it is possible that homologs of neural HD proteins expressed in other tissues would also interact with Groucho and GliR to mediate correct Shh signaling interpretation.

Importantly, the expression along entire D/V axis of the CRM<sup>Nkx6.1</sup> containing two inactivated HBSs is dependent on GBS1 and SBS2 and inhibited by co-expression of Ptc1<sup>Δloop2</sup>, a construct that cell-autonomously inhibits Shh signal transduction (Briscoe et al., 2001). These observations argue that GliA-SoxB1 complexes uniformly activate *Nkx6.1* expression along the entire D/V axis of the neural tube, whereas the GliR-HD repressive complexes translate the graded Shh-activity into the precise spatial pattern. Correspondingly, the induction by GliA-SoxB1 and repression by GliR-HD define core mechanisms that prospectively govern expression of all Shh-target genes in the developing CNS.

### 3.5. Cell-intrinsic modulation of Shh strength: concentration-independent step-function

Most current models of Shh interpretation exploit graded effects of the signaling, however in mice lacking *Ptc1* multiple ventral domains are specified despite the fact that cells are unable to detect differences in the Shh concentration (Figure 5) (Motoyama et al., 2003). Moreover, the loss of ventral domains observed in mouse mutants with abrogated GliA activity, *Shh*<sup>-/-</sup> and *Smo*<sup>-/-</sup> mutants, is partially restored upon further removal of Gli input, that is the Gli3 mediated repression (Figure 5) (Persson et al., 2002). These observations raise the possibility that some aspects of Shh induced spatial patterning occur in a concentration independent manner.

Dorsal, intermediate and ventral parts of the neural tube respond to Shh with varied competence (Ruiz i Altaba et al., 1995) and in addition, ventral progenitors adapt to ongoing Shh signaling with time (Dessaud et al., 2010; Dessaud et al., 2007). These results suggest that intrinsic mechanisms can adjust the interpretation of Shh pathway. In Paper II we explore this possibility by addressing the role of the HD proteins Pax6, Nkx2.2 and Nkx2.9 in modulating the intracellular strength of Shh signaling.

#### 3.4.1. Reshaping of the intrinsic ratio of GliA:GliR

In Paper II we show that both the FP and the p3 domains are formed from progenitors that initially express Nkx2.2 protein. Early Nkx2.2 expression co-localizes with *Foxa2* marking the induction of the presumptive FP domain. Subsequently, Nkx2.2 expression expands dorsally while being down-regulated in the *Foxa2* expressing cells, whereupon two separate domains, p3 and FP, are delineated. Interestingly, whereas the *Nkx2.2* and *Nkx2.9* single mutants show mild reduction of V3 cells or no phenotype, respectively (Briscoe et al., 1999; Pabst et al., 2003), in *Nkx2.2;Nkx2.9* double mutants p3 progenitors do not form and the expression of *Foxa2* and the size of FP is severely reduced. These observations reveal an unexpected requirement of Nkx2 proteins for the establishment of FP cells in addition to V3 cell fate. To test if the activity of Nkx2 proteins is sufficient to specify the FP fate, we overexpressed Nkx2.2 and Nkx2.9 proteins in the chick neural tube and observed ectopic induction of *Foxa2* followed by induction of Shh in both experiments, indicating an acquirement of FP identity. Strikingly, this effect of Nkx2 is restricted to early onset of Nkx2.2 activity as the overexpression of Nkx2.2 at later developmental time instead leads to ectopic induction of V3 cell fate.

Importantly, no ectopic *Foxa2* activation is observed when Nkx2.2 is co-expressed with repressor form of Gli3 or with *Ptc1*<sup>Δloop2</sup>, which attenuates Shh signal transduction cell autonomously, suggesting that the induction of FP by Nkx2.2 is dependent on activation of the Shh pathway. We further investigated the effect of Nkx2.2 on intracellular Shh signaling and found that overexpression of Nkx2.2 is able to repress *Gli3*, the main repressor in the Shh pathway. Moreover, Nkx2.2 also ectopically activated CRM<sup>Nkx2.2</sup> and this activation was dependent on an intact GBS. Collectively,

these results argue that Nkx2 proteins are able to modulate intrinsic cellular responses to Shh by increasing Gli activity.

Notably, other groups have revealed that Foxa2, upon induction, starts to attenuate the intracellular Shh signaling response and to exert repressive effects on *Nkx2.2* (Cruz et al. 2010, Ribes et al. 2010). Furthermore, Foxa2 and its related family member Foxa1 have been shown to inhibit Gli2 expression in the ventral midbrain possibly by a direct binding to putative CRMs surrounding *Gli2* gene (Mavromatakis et al., 2011). Thus, Foxa2 is able to modulate intrinsic Gli activity negatively, which is a prerequisite for the establishment of FP identity (Ribes et al. 2010).

Furthermore, in Paper II we investigate if other HD proteins can sensitize Shh responsiveness in the neural tube. Pax6 is expressed in the complementary domain dorsal to the Nkx2 domain in the developing spinal cord in which high levels of Pax6 expression correlate with a low probability of adopting the FP-fate in response to Shh (Ericson et al., 1997b; Ruiz i Altaba et al., 1995). We found that forced expression of Pax6 protein inhibited Foxa2 expression and caused dorsal expansion of the intermediate progenitor genes; *Dbx1* and *Dbx2*. Strikingly, ectopic expression of *Gli3* was observed in these experiments, arguing that Pax6 is able to intrinsically reduce intracellular Shh strength by up-regulating *Gli3* expression.

Collectively these experiments suggest that the feedback activity exerted by HD proteins on expression levels of Gli genes provides an intrinsic, gradient-independent regulation of Shh activity. In Paper I we show that in the neural tube, GliR levels are instructive in regulating the expression patterns of the long-range Shh-target genes, whereas at short-range cells interpret GliA:GliR level differences. Thus Pax6/Nkx2.2-mediated regulation of Gli3 expression, the main Shh-pathway repressor, is directly involved in enhancing these effects. Pax6, a known activator, enhances *Gli3* expression, thereby making more full-length Gli3 protein accessible for proteolytic cleavage at varying distances from Shh source. In contrast, the repressor activities of Nkx2 proteins inhibit expression of *Gli3* in the most ventral region of the neural tube, thus changing the GliA:GliR ratio and allowing GliA to accumulate to levels sufficient for the induction of FP and p3 cells. Strikingly, the complete loss of Shh<sup>+</sup> FP cells in Gli2 mutants can be restored when also Pax6 expression is eliminated as shown by FP rescue in the *Pax6;Gli2* double mutants (Paper II). These results strongly argue that Pax6 and the opposing activity of Nkx2 can sensitize the output of graded Shh signaling thus allowing the neighboring cells to activate different genetic programs largely independent of extracellular concentration of Shh. Moreover, neural-specific *Gli3* enhancers contain putative HD binding sites, suggesting that regulation by Pax6 and Nkx2 might be on a direct transcriptional level (Abbasi et al., 2007).

### **3.6. Temporal alterations of cellular competence**

Both V3 and FP cells are generated from the Nkx2<sup>+</sup> domain. Remarkably, the induction of FP identity precedes the establishment of p3 domain, even though the ambient concentration of Shh is lower at that time (Chamberlain et al., 2008). If FP cells



required higher Shh signal, they would be induced after the dorsally adjacent p3 domain when Shh amplitude is higher, hence a simple Shh gradient model cannot account for this temporal switch. Sensitization to the Shh signal is also unlikely to explain the switch in progenitor potential as they both arise from *Nkx2*<sup>+</sup> cells. Therefore we exploited alternative changes in the cellular competence of *Nkx2* expressing progenitors.

FP cells have glia-like traits and are specified prior to the induction of proneural genes in the neural tube allowing us to speculate that a temporal switch in cellular neurogenic potential could be responsible for spatial acquirement of p3 domain dorsal to FP. In Paper II, we test this hypothesis by overexpressing proneural genes in the FP region and find that ectopic expression of *Ngn2* or *Ngn3* represses FP fate and activates ectopic p3 program. Furthermore, when co-expressed with *Nkx2.2*, *Ngn2/3* are able to suppress *Nkx2.2*-mediated ectopic induction of FP. These results argue that cell intrinsic induction of neurogenic factors constrains spatial induction of FP, in agreement with our results showing that induction of FP by *Nkx2.2* is dependent on developmental stage. Moreover, our results are consistent with several other studies showing that the induction of FP is restricted to early embryonic stages (Patten and Placzek, 2002; Ribes et al., 2010). Succinctly, the temporal changes in cellular competence by acquisition of neurogenic potential provide an intrinsic mechanism that determines the fate choice in the ventral most region of the neural tube.

In the dorsal neural tube many proneural genes are expressed at early developmental stages and function as cell-fate determining TF. Consequently, in addition to these functions, *Math1* and *Ngn1/2* might be involved in adjusting intrinsic cellular properties that separate the RP from the two dorsalmost neurogenic progenitor domains.

### **3.7. Neural-specific interpretation of the Shh pathway**

Shh is a representative of a small number of signaling pathways deployed during embryogenesis to regulate patterning and growth of various tissues. Each of these pleiotropic pathways directs diverse responses in distinct tissues by activating specific sets of target genes, whereas the same genes are silent in adjacent tissue exposed to the same signal. Paper III deals with the intriguing question of how neural tissue-specific outcomes arise from these common signaling pathways.

The functional characterization of Shh regulated CRMs performed in Paper I showed that all CRMs contain functional SBS and that the activation of CRMs associated with class II genes is dependent on cooperation of GliA and SoxB1 proteins. In Paper III, we performed further bioinformatic searches looking for co-localization of GBS and SBS on elements bound by p300, a transcriptional co-activator that has been shown to accurately predict tissue specific enhancer activity (Visel et al., 2009). We compared neural tissue with limb tissue and found a significant overrepresentation of SBSs within 50bp from GBSs in neural elements associated with p300. SoxB1 are broadly expressed in the CNS but not in most other tissues and we hypothesized that they could contribute to the neuronal-specific selection of Shh-targets.

The developing limb tissue does not express SoxB1 proteins and we therefore tested if SoxB1 expression could endow limb bud cells with the competence to activate neural-specific Shh-target genes. Strikingly, miss-expression of Sox2 or Sox3 in combination with strong activation of Shh signaling (SmoM2 overexpression) in the limb bud resulted in ectopic activation of neural-specific *Nkx2.2*, *Nkx6.1*, *Nkx6.2* and *Olig2* genes as well as their respective CRMs. Notably, miss-expression of Sox2, Sox3 or SmoM2 alone did not induce class II genes, implying that activation of Shh signaling without SoxB1 input is not sufficient to activate neural-specific Shh-targets in the developing limbs. Moreover, the ectopic Sox3/SmoM2-mediated activation of the class II CRMs depended on intact SBSs, indicating a direct requirement for SoxB1 binding to the individual CRMs. Sox3 miss-expression resulted in negligible induction of the neural marker *Sox1*, whereas the expression of the mesodermal marker *dHand* was maintained in SoxB1-expressing cells, arguing against the possibility that the ectopic activation of class II genes reflects a reprogramming of mesodermal cells into bona fide neural progenitors. Although our experiments do not exclude an epigenetic role for SoxB1 proteins, the data strongly implies that the tissue-specific expression of Shh-regulated genes is primarily determined by the combinatorial activity of SoxB1 and Gli at the transcriptional level.

The general tissue-non-specific Shh-target gene, *Ptc1*, is flanked by several Gli-bound regulatory regions suggesting that multiple GBSs might contribute to the sensitivity of *Ptc1* to Shh (Vokes et al., 2007). Unexpectedly, some of these regions exhibit a certain degree of tissue-specificity opening the possibility that even *Ptc1* expression in fact may be controlled by cell-type-specific response elements. However, we have tested the in vitro transcriptional activity of the best-characterized *Ptc1* regulatory element that contains one functional high affinity GBS (TGGGTGGTC) (Agren et al., 2004) and shows strong activity in the CNS as well as other tissues (Vokes et al., 2007) and observed that GliA mediated activation of CRM<sup>Ptc1</sup> is not affected by the addition of SoxB1 (data not shown). Moreover, strong ectopic induction of *Ptc1* in the limb bud was detected upon overexpression of SmoM2 independent of co-electroporation with SoxB1. These results suggest that Shh regulation of *Ptc1* expression is independent of SoxB1 co-activation and further support our model of SoxB1-mediated neuronal-specific selection of Shh target genes.

### 3.8. Neural-specific interpretation of other pleiotropic pathways

We extended our study in Paper III and investigated if SoxB1 binding underlies neural-specific interpretation of other pleiotropic pathways. We focused on RA and BMP signaling since they are important for patterning of the intermediate and the dorsal parts of the neural tube, respectively, and also regulate development of the limb tissue.

Examination of the CRMs active in the intermediate neural tube, CRM<sup>Dbx1</sup> and CRM<sup>Dbx2</sup>, revealed that each CRM in addition to several SBSs also contains conserved nuclear receptor binding sites (NRBS) resembling RAR/RXR binding motifs (RARE). We found that the activity of these CRMs in the neural tube is critically dependent on

both SBSs and NRBS and that in vitro transcriptional assays, RA is able to activate CRM<sup>Dbx1</sup> synergistically with SoxB1 in a SBS and NRBS dependent manner. These results argue for a requirement of direct binding of SoxB1 and RAR/RXR to the CRM<sup>Dbx1</sup> to induce *Dbx1* by RA signaling in a neural tissue. In addition, mis-expression of Sox3 in limb bud tissue was sufficient to induce *Dbx1*, *Dbx2* and *Pax6* genes as well as the CRM<sup>Dbx1</sup>. Furthermore, analysis of conserved RAREs in p300-bound elements revealed an overrepresentation of SBSs located in proximity to RAREs in elements active in neural tissue compared to limb tissue, suggesting that a broad range of neural-specific RA-targets depends on SoxB1 binding for activation.

The BMP pathway regulates patterning of the dorsal neural tube including expression of *Msx* and *Gsh* genes. We isolated a CRM that recapitulates *Msx1* expression in the neural tube and show that it requires intact SBS and Smad binding sites (SmSB) for neural activity. However, constitutively active form of BMPRI (Alk-2<sup>CA</sup>) was able to, both in neural tube and the limb bud, induce ectopic expression of wild-type CRM<sup>Msx1</sup> and the CRM<sup>Msx1</sup> containing inactive SBS, whereas the CRM<sup>Msx1</sup> with inactive SmSB was not activated. These results suggest that SoxB1 promotes neural expression of *Msx1* but is not absolutely required for Smad-mediated induction of *Msx1*. Notably, *Msx1* is endogenously expressed both by neural tube and limbs, which could provide an explanation for the lower dependency on co-activation by SoxB1. By contrast, the CNS-active *Gsh1* that is not endogenously expressed in the limb tissue, required cooperative activation by the BMP pathway and SoxB1 proteins to be ectopically induced in the limb bud, implying that at least a subset of neural-specific BMP targets requires cooperative activation by SoxB1 proteins.

### 3.9. Genome-wide prediction of neural-specific morphogen targets

The functional association between SBS and GBS of known neural-specific patterning genes led us to examine if other genes could be regulated by the same transcriptional logic. The furthest distance between a functional GBS and the nearest conserved SBS in the CRMs associated with Shh-induced class II genes was 36 bp. We performed a genome-wide search for non-coding elements conserved between mouse, human, and opossum that contained co-located consensus GBS and SBS with maximum distance of 36bp. We identified 83 presumptive CRMs with these criteria and the number of positive regions decreased with increasing distance between conserved GBS and SBS. Furthermore, a survey of databases and the literature indicated that genes encoding TFs linked to identified GBS-SBS elements have a higher probability of being expressed in the ventral neural tube as compared to the posterior limb bud. A similar genome-wide search between mouse and chick genome identified 45 putative CRM out of which we were able to obtain 15 functional RNA-probes. Expression analysis of this set of genes revealed that 87% of genes showed a clear ventral bias of expression in the neural tube and strikingly, most of these genes were ectopically activated in limb bud tissue in response to Sox3 and SmoM2 expression. Based on these experiments, we propose that

the co-localization of SBS and GBS provides a general transcriptional mechanism that underlies the tissue-specific activation of Shh-regulated genes in the developing CNS.

In addition, a complementary genome-wide analysis identified 545 RAREs conserved between mouse and chick. Importantly, co-localized SBSs within 50bp from RAREs were found in regions nearby genes significantly enriched for functions in neural development compared to limb development. In contrast, genes lying nearby RAREs located further than 50bp away from an SBS showed essentially no difference in functional annotation between the two tissues implying that an SBS-RARE transcriptional code determines neural-specific RA-target gene activation.

Collectively, our data provide evidence for a functional integration of SoxB1 proteins and terminal mediators of Shh, RA, and BMP pathways at the CRM level, where the proximity of binding sites is a deciding element. Thus SoxB1-mediated activation mechanism offers a general transcriptional strategy for the neural-specific interpretation of pleiotropic signals during embryogenesis.

Similarly, GBSs present in the limb bud elements bound by p300 showed a clear bias for association with HBS sites, within which the binding site for Prrx2 protein had the highest score. Prrx1 and Prrx2 proteins have been shown to be important for limb development and skeletogenesis, where their functions have been linked both to activation and repression (Lu et al., 1999; Lu et al., 2011). It is intriguing to speculate if these proteins could be limb bud tissue-specific co-activators of Shh signaling allowing gradient-independent GliA activation of target genes. Moreover, given that repression by Gli3 appears to be an instructive mechanism in establishing the long-range A/P axis of the developing limb, it will be interesting to alternatively investigate if Prrx proteins could serve as limb-specific Gli3 co-repressors.

### **3.10. SoxB1 mechanism**

SoxB1 TFs are critically required for GliA-mediated induction of Shh-target genes in neural tissue and for neural-specific interpretation of other pleiotropic pathways. However, SoxB1 have low *trans*-activating potential (Kamachi et al., 2000). Therefore one likely function of these proteins would be to stabilize GliA, Smad or RAR-RXR binding to the CRMs, rendering it insensitive to the binding site affinity. The binding of SoxB1 to SBSs causes DNA bending which could provide a mechanistic logic behind facilitated GliA, Smad and RAR-RXR binding and bring additional co-activators in physical proximity. Alternatively, protein interactions between SoxB1 and their specific partners could be efficiently recruiting RNA-polymerase II complexes, in consistence with the observation that Sox2 recruits p300 protein to the Fibroblast Growth Factor 4 enhancer (Nowling et al., 2003).

Additionally, SoxB1 have also been implicated in epigenetic control of gene expression (Bergsland et al., 2011; Lee et al., 2006a). However, prior to the commitment to neural lineage the putative neural-specific regulatory elements highlighted by the genomic Sox2 or Sox3 binding display bivalent chromatin methylation marks, suggestive of partially open and partially closed chromatin state. These observations imply that

binding of SoxB1 to these CRMs is not sufficient to completely open the chromatin structure (Bergsland et al., 2011). Nevertheless, analyses of the chromatin state of SoxB1 bound neural enhancers in comparison to the same but inactive enhancers in the limb tissue would make an interesting experiment, particularly in addition to modulating Shh pathway in these cells. In the limb tissue the repressive function of Gli3 has been linked to chromatin silencing via Ski co-repressor that binds to Gli3 and recruits the histone deacetylase complexes (HDAC) (Dai et al., 2002). Since Ski is expressed by neural cells (Baranek and Atanasoski, 2012), a similar mechanism could also be operating in the neural tissue. Moreover, Gli proteins have been proposed to, during neuronal development, interact and recruit Brg, an ATP-dependent chromatin-remodeling factor, to the Gli regulatory regions (Zhan et al., 2011). The authors proposed that Brg interaction with Gli3 influences Gli3-mediated repression. Interestingly, they also suggested that Brg is required for Gli-mediated transcriptional activation, a mechanism that is at least partially facilitated by recruiting HDAC to the regulatory elements. Surprisingly, HDAC, a regulator of chromatin condensation (Milon et al., 2012), can induce posttranslational modification of Gli1 and Gli2 proteins that enhances their activity (Canettieri et al., 2010) although, it is unclear to which extent chromatin remodeling is involved in these mechanisms. Collectively, these observations raise the possibility that both SoxB1- and Gli-induced chromatin remodeling might play a significant role in the transcriptional regulation of Shh target genes.

The members of several Sox families are expressed in various tissues during embryogenesis, some of which also receive Shh, BMP and/or RA signal. For example SoxE group members, Sox9 and Sox10, are pivotal in neural crest development and chondrogenesis requires Sox9 expression (Kiefer, 2007). Even in the developing neural tube the activity of SoxE proteins is required for neuronal to glial progenitor switch (Sox8 and Sox9) and oligodendrocyte differentiation (Sox8 and Sox10) (Kiefer, 2007). Moreover, the HMG domain is highly conserved among Sox factors and in vitro DNA binding studies show no significant differences in sequence recognition among family members (Kamachi et al., 2000). Can then other Sox groups serve as the temporal and tissue-specific partners of Gli, RXR and Smad proteins by binding to the same SBSs?

Interestingly, the regions outside the HMG box are highly diversified and in vivo, Sox proteins show clear distinctions between their binding targets (Miyagi et al., 2009; Zhao and Koopman, 2012). The diversity of the non-HMG domains of Sox proteins implies that the selection of binding partners will differ among Sox sub-groups. Accordingly, different Sox proteins have been shown to interact with different binding partners to activate distinct target genes (Kamachi et al., 2000). For instance, Sox2 cooperates with Pax6 to activate the  $\delta$ -crystallin enhancer and this cooperation enhances the binding stability of the individual proteins (Kamachi et al., 2001). Cooperation with Pax6 appears to be restricted to SoxB1 as Sox9 fails to activate the same targets in transcriptional assays even though in isolation Sox9 binds to the target sequences with the same affinity as SoxB1 (Kamachi et al., 1999). These observations raise the possibility that Gli, Smad or RAR/RXR proteins bind selectively SoxB1

however, further studies, for example by overexpression of members of SoxE with SmoM2 in the limb bud, are needed to verify this hypothesis.

Furthermore, genome-wide analyses of BMP pathway revealed that master regulators of lineage committing properties direct the binding of Smad1 to specific target genes that are active in only specific cell-lineages (Trompouki et al., 2011). These studies have observed that upon differentiation or miss-expression of alternative lineage master regulators in hematopoietic progenitors, partial relocation of Smad1 binding occurs. Thereby this study proposes that alternations in expression of master lineage regulators can dictate the binding of pathway-terminal TF. It is feasible to expect that miss-expression of SoxB1 in these cell lineages would also globally change Smad1 binding such that it is directed to the neural specific CRMs. It is also feasible to expect that miss-expression of SoxB1 in any tissue regulated by Shh or RA signaling would make the same global change of directing GliA or RAR/RXR binding to neural-specific CRMs.

## 4. CONCLUDING REMARKS

All developmental processes require coordinated interaction between extracellular signaling molecules and intracellular transcriptional regulators to strictly control gene expression and functional output. During the development of the CNS, morphogens are crucial for setting up spatial patterns of gene expression, which are further refined by intrinsic changes in cellular competence. Moreover, these developmental mechanisms intersect at the regulatory elements of patterning genes and exert combinatorial control of gene expression.

This thesis focuses on the roles of Shh signaling on neural subtype specification and elucidates a mechanism whereby GliR-mediated spatial regulation of ventrally expressed neuronal genes acts at long-range, whereas GliA and SoxB1 uniformly activate gene-associated CRMs in a largely Shh concentration-independent manner. We further show that cross-repressive mechanisms refine the spatial expression through cooperative binding with GliR to the same CRMs. In addition, Nkx2 and Pax6 TFs sensitize cells to Shh signaling by influencing Gli3 expression. Thereby, Nkx2 and Pax6 are involved in feedforward amplification or feedback antagonizing mechanisms, respectively, which adjust intracellular Gli activity and thereby influence their own CRM activity.

Furthermore, BMPs and RA are important regulators of the spatial gene expression in the dorsal and intermediate spinal cord. Our experiments suggest that the downstream transcriptional mediators of RA and BMP signaling require interaction with SoxB1 proteins on the regulatory elements of target genes to achieve neural-specific activation. Moreover, a subset of these CRMs is also regulated by Shh/Gli signaling.

As mechanisms governing specific developmental events continue to be identified, a greater challenge in developmental biology will be to integrate all regulatory information into models that allow visualization of combinatorial effects and thus prediction of normal development and disease. Gene regulatory networks (GRNs) are emerging as such models with potential to integrate all causative links between the regulatory molecules converging on the CRMs and the target gene outputs (Davidson and Levine, 2008).

In our model of neural-specific GRNs, SoxB1 and transcriptional mediators of Shh, BMP and RA signaling are the central factors required to translate graded signals into regional gene expression patterns. An example of how the characterized mechanisms establish a regulatory network is the interactions between *Msx1*, *Dbx1* and *Nkx6.1*. *Msx1* is induced by the cooperative activity of SoxB1 and Smads in the dorsal neural tube, where in combination with GliR it represses *Dbx1* and *Nkx6.1*. By contrast, SoxB1 and RA signaling activate *Dbx1* in the intermediate spinal cord, where *Dbx1* represses *Msx1* and *Nkx6.1*. The regulatory loop is completed in the ventral neural tube

where SoxB1 and GliA synergistically induce *Nkx6.1*, which inhibits *Dbx1* and *Msx1* expression. We provide evidence that most of these mechanisms are transcriptional events occurring directly at the CRMs associated with *Msx1*, *Dbx1* and *Nkx6.1*. Thereby, the individual CRMs interlink the mechanisms operating within the GRNs. Moreover, the GRNs are repeatedly deployed in different cellular contexts. SoxB1 regulated neuronal networks are under the influence of different regulators in other tissues, but can be redeployed by limb mesodermal cells simply by misexpressing one central activator of the GRN. Accordingly, the SoxB1 transcriptional code provides not only a strategy for induction of GRNs that drive morphogen interpretation and determine the positional identity of cells, but also for the tissue-specific selection of target genes.

Moreover, large-scale analyses have recently suggested that significant portions of the human genome have regulatory potential (Dunham et al., 2012). If accurately understood, the mechanisms encoded in these sequences would create a profound basis for the establishment of novel and more efficient therapies, such as tissue engineering, aimed for treatment of human diseases. Moreover, the high quantity of regulatory sequences highlights the potential gene changes associated with diseases to reside within the regulatory aspects of gene expression in contrast to protein coding alterations (Betts et al., 2012; VanderMeer and Ahituv, 2011; Ward and Kellis, 2012) opening a new exciting era of disease related research.



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## REFERENCES

- Abbasi, A.A., Paparidis, Z., Malik, S., Goode, D.K., Callaway, H., Elgar, G., and Grzeschik, K.H. (2007). Human GLI3 intragenic conserved non-coding sequences are tissue-specific enhancers. *PloS one* 2, e366.
- 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.
- Allan, D.W., and Thor, S. (2003). Together at last: bHLH and LIM-HD regulators cooperate to specify motor neurons. *Neuron* 38, 675-677.
- 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 & development* 21, 1244-1257.
- Alvarez Martinez, C.E., Binato, R., Gonzalez, S., Pereira, M., Robert, B., and Abdelhay, E. (2002). Characterization of a Smad motif similar to *Drosophila* mad in the mouse *Msx 1* promoter. *Biochemical and biophysical research communications* 291, 655-662.
- Apidianakis, Y., Grbavec, D., Stifani, S., and Delidakis, C. (2001). Groucho mediates a Ci-independent mechanism of hedgehog repression in the anterior wing pouch. *Development* 128, 4361-4370.
- 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. *Developmental cell* 6, 103-115.
- Baranek, C., and Atanasoski, S. (2012). Modulating epigenetic mechanisms: the diverse functions of Ski during cortical development. *Epigenetics* 7, 676-679.
- Bergsland, M., Ramskold, D., Zaouter, C., Klum, S., Sandberg, R., and Muhr, J. (2011). Sequentially acting Sox transcription factors in neural lineage development. *Genes & development* 25, 2453-2464.
- Bertrand, N., Castro, D.S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nature reviews Neuroscience* 3, 517-530.
- Betts, J.A., French, J.D., Brown, M.A., and Edwards, S.L. (2012). Long-range transcriptional regulation of breast cancer genes. *Genes Chromosomes Cancer*.
- Blitz, I.L., and Cho, K.W. (2009). Finding partners: how BMPs select their targets. *Developmental dynamics : an official publication of the American Association of Anatomists* 238, 1321-1331.
- Bond, A.M., Bhalala, O.G., and Kessler, J.A. (2012). The dynamic role of bone morphogenetic proteins in neural stem cell fate and maturation. *Developmental neurobiology* 72, 1068-1084.
- 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. *Nature neuroscience* 8, 297-304.
- Bowers, M., Eng, L., Lao, Z., Turnbull, R.K., Bao, X., Riedel, E., Mackem, S., and Joyner, A.L. (2012). Limb anterior-posterior polarity integrates activator and repressor functions of GLI2 as well as GLI3. *Developmental biology* 370, 110-124.
- 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. *Molecular cell* 7, 1279-1291.
- Briscoe, J., Pierani, A., Jessell, T.M., and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* 101, 435-445.
- 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.
- Brugger, S.M., Merrill, A.E., Torres-Vazquez, J., Wu, N., Ting, M.C., Cho, J.Y., Dobias, S.L., Yi, S.E., Lyons, K., Bell, J.R., *et al.* (2004). A phylogenetically conserved cis-regulatory module in the *Msx2*

promoter is sufficient for BMP-dependent transcription in murine and *Drosophila* embryos. *Development* *131*, 5153-5165.

Bylund, M., Andersson, E., Novitsch, B.G., and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nature neuroscience* *6*, 1162-1168.

Canettieri, G., Di Marcotullio, L., Greco, A., Coni, S., Antonucci, L., Infante, P., Pietrosanti, L., De Smaele, E., Ferretti, E., Miele, E., *et al.* (2010). Histone deacetylase and Cullin3-REN(KCTD11) ubiquitin ligase interplay regulates Hedgehog signalling through Gli acetylation. *Nat Cell Biol* *12*, 132-142.

Cayuso, J., Ulloa, F., Cox, B., Briscoe, J., and Marti, E. (2006). The Sonic hedgehog pathway independently controls the patterning, proliferation and survival of neuroepithelial cells by regulating Gli activity. *Development* *133*, 517-528.

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.

Chen, J.K., Taipale, J., Young, K.E., Maiti, T., and Beachy, P.A. (2002). Small molecule modulation of Smoothened activity. *Proceedings of the National Academy of Sciences of the United States of America* *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 & development* *18*, 641-659.

Chen, Y., Sasai, N., Ma, G., Yue, T., Jia, J., Briscoe, J., and Jiang, J. (2011). Sonic Hedgehog dependent phosphorylation by CK1 $\alpha$  and GRK2 is required for ciliary accumulation and activation of smoothened. *PLoS biology* *9*, e1001083.

Cheung, H.O., Zhang, X., Ribeiro, A., Mo, R., Makino, S., Puvion-Rodan, V., Law, K.K., Briscoe, J., and Hui, C.C. (2009). The kinesin protein Kif7 is a critical regulator of Gli transcription factors in mammalian hedgehog signaling. *Science signaling* *2*, ra29.

Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., and Beachy, P.A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* *383*, 407-413.

Cinnamon, E., and Paroush, Z. (2008). Context-dependent regulation of Groucho/TLE-mediated repression. *Curr Opin Genet Dev* *18*, 435-440.

Colas, J.F., and Schoenwolf, G.C. (2001). Towards a cellular and molecular understanding of neurulation. *Developmental dynamics : an official publication of the American Association of Anatomists* *221*, 117-145.

Cooper, A.F., Yu, K.P., Brueckner, M., Brailey, L.L., Johnson, L., McGrath, J.M., and Bale, A.E. (2005). Cardiac and CNS defects in a mouse with targeted disruption of suppressor of fused. *Development* *132*, 4407-4417.

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.

Dai, P., Shinagawa, T., Nomura, T., Harada, J., Kaul, S.C., Wadhwa, R., Khan, M.M., Akimaru, H., Sasaki, H., Colmenares, C., *et al.* (2002). Ski is involved in transcriptional regulation by the repressor and full-length forms of Gli3. *Genes & development* *16*, 2843-2848.

Davidson, E.H., and Levine, M.S. (2008). Properties of developmental gene regulatory networks. *Proceedings of the National Academy of Sciences of the United States of America* *105*, 20063-20066.

Denef, N., Neubuser, D., Perez, L., and Cohen, S.M. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. *Cell* *102*, 521-531.

Dessaud, E., McMahon, A.P., and Briscoe, J. (2008). Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. *Development* *135*, 2489-2503.

Dessaud, E., Ribes, V., Balaskas, N., Yang, L.L., Pierani, A., Kicheva, A., Novitsch, B.G., Briscoe, J., and Sasai, N. (2010). Dynamic assignment and maintenance of positional identity in the ventral neural tube by the morphogen sonic hedgehog. *PLoS biology* *8*, e1000382.

- 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.
- Dorn, K.V., Hughes, C.E., and Rohatgi, R. (2012). A Smoothed-Evc2 complex transduces the Hedgehog signal at primary cilia. *Developmental cell* *23*, 823-835.
- Dunham, I., Kundaje, A., Aldred, S.F., Collins, P.J., Davis, C.A., Doyle, F., Epstein, C.B., Frietze, S., Harrow, J., Kaul, R., *et al.* (2012). An integrated encyclopedia of DNA elements in the human genome. *Nature* *489*, 57-74.
- 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.
- Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V., and Jessell, T.M. (1997a). Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube. *Cold Spring Harbor symposia on quantitative biology* *62*, 451-466.
- Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T.M., and Briscoe, J. (1997b). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* *90*, 169-180.
- Grabel, L. (2012). Prospects for pluripotent stem cell therapies: into the clinic and back to the bench. *J Cell Biochem* *113*, 381-387.
- Graham, V., Khudyakov, J., Ellis, P., and Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. *Neuron* *39*, 749-765.
- Guerrero, I., and Chiang, C. (2007). A conserved mechanism of Hedgehog gradient formation by lipid modifications. *Trends in cell biology* *17*, 1-5.
- Hallikas, O., and Taipale, J. (2006). High-throughput assay for determining specificity and affinity of protein-DNA binding interactions. *Nat Protoc* *1*, 215-222.
- Hardison, R.C., and Taylor, J. (2012). Genomic approaches towards finding cis-regulatory modules in animals. *Nature reviews Genetics* *13*, 469-483.
- 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. (2005). Cilia and Hedgehog responsiveness in the mouse. *Proceedings of the National Academy of Sciences of the United States of America* *102*, 11325-11330.
- Ingham, P.W., and Placzek, M. (2006). Orchestrating ontogenesis: variations on a theme by sonic hedgehog. *Nature reviews Genetics* *7*, 841-850.
- 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. *Nature reviews Genetics* *1*, 20-29.
- Kamachi, Y., Cheah, K.S., and Kondoh, H. (1999). Mechanism of regulatory target selection by the SOX high-mobility-group domain proteins as revealed by comparison of SOX1/2/3 and SOX9. *Molecular and cellular biology* *19*, 107-120.
- Kamachi, Y., Uchikawa, M., and Kondoh, H. (2000). Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet* *16*, 182-187.
- Kamachi, Y., Uchikawa, M., Tanouchi, A., Sekido, R., and Kondoh, H. (2001). Pax6 and SOX2 form a co-DNA-binding partner complex that regulates initiation of lens development. *Genes & development* *15*, 1272-1286.
- Keady, B.T., Samtani, R., Tobita, K., Tsuchya, M., San Agustin, J.T., Follit, J.A., Jonassen, J.A., Subramanian, R., Lo, C.W., and Pazour, G.J. (2012). IFT25 links the signal-dependent movement of Hedgehog components to intraflagellar transport. *Developmental cell* *22*, 940-951.
- Kiefer, J.C. (2007). Back to basics: Sox genes. *Developmental dynamics : an official publication of the American Association of Anatomists* *236*, 2356-2366.

- Kriegstein, A., and Alvarez-Buylla, A. (2009). The glial nature of embryonic and adult neural stem cells. *Annual review of neuroscience* 32, 149-184.
- Kriks, S., Lanuza, G.M., Mizuguchi, R., Nakafuku, M., and Goulding, M. (2005). Gsh2 is required for the repression of Ngn1 and specification of dorsal interneuron fate in the spinal cord. *Development* 132, 2991-3002.
- Lander, A.D. (2007). Morpheus unbound: reimagining the morphogen gradient. *Cell* 128, 245-256.
- Larkins, C.E., Aviles, G.D., East, M.P., Kahn, R.A., and Caspary, T. (2011). Arl13b regulates ciliogenesis and the dynamic localization of Shh signaling proteins. *Molecular biology of the cell* 22, 4694-4703.
- Lee, T.I., Jenner, R.G., Boyer, L.A., Guenther, M.G., Levine, S.S., Kumar, R.M., Chevalier, B., Johnstone, S.E., Cole, M.F., Isono, K., *et al.* (2006a). Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell* 125, 301-313.
- Lee, Y., Miller, H.L., Russell, H.R., Boyd, K., Curran, T., and McKinnon, P.J. (2006b). Patched2 modulates tumorigenesis in patched1 heterozygous mice. *Cancer research* 66, 6964-6971.
- Lei, Q., Jeong, Y., Misra, K., Li, S., Zelman, A.K., Epstein, D.J., and Matisse, M.P. (2006). Wnt signaling inhibitors regulate the transcriptional response to morphogenetic Shh-Gli signaling in the neural tube. *Developmental cell* 11, 325-337.
- Lei, Q., Zelman, A.K., Kuang, E., Li, S., and Matisse, 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.
- Liem, K.F., Jr., He, M., Ocbina, P.J., and Anderson, K.V. (2009). Mouse Kif7/Costal2 is a cilia-associated protein that regulates Sonic hedgehog signaling. *Proceedings of the National Academy of Sciences of the United States of America* 106, 13377-13382.
- Liem, K.F., Jr., Jessell, T.M., and Briscoe, J. (2000). Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. *Development* 127, 4855-4866.
- Litingtung, Y., Dahn, R.D., Li, Y., Fallon, J.F., and Chiang, C. (2002). Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. *Nature* 418, 979-983.
- Litingtung, Y., Lei, L., Westphal, H., and Chiang, C. (1998). Sonic hedgehog is essential to foregut development. *Nature genetics* 20, 58-61.
- Liu, A., and Niswander, L.A. (2005). Bone morphogenetic protein signalling and vertebrate nervous system development. *Nature reviews Neuroscience* 6, 945-954.
- Liu, Y., Helms, A.W., and Johnson, J.E. (2004). Distinct activities of Msx1 and Msx3 in dorsal neural tube development. *Development* 131, 1017-1028.
- Lu, M.F., Cheng, H.T., Lacy, A.R., Kern, M.J., Argao, E.A., Potter, S.S., Olson, E.N., and Martin, J.F. (1999). Paired-related homeobox genes cooperate in handplate and hindlimb zeugopod morphogenesis. *Developmental biology* 205, 145-157.
- Lu, X., Beck, G.R., Jr., Gilbert, L.C., Camalier, C.E., Bateman, N.W., Hood, B.L., Conrads, T.P., Kern, M.J., You, S., Chen, H., *et al.* (2011). Identification of the homeobox protein Prx1 (MHox, Prrx-1) as a regulator of osterix expression and mediator of tumor necrosis factor alpha action in osteoblast differentiation. *J Bone Miner Res* 26, 209-219.
- 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.
- Maden, M. (2006). Retinoids and spinal cord development. *Journal of neurobiology* 66, 726-738.
- Mahony, S., Mazzoni, E.O., McCuine, S., Young, R.A., Wichterle, H., and Gifford, D.K. (2011). Ligand-dependent dynamics of retinoic acid receptor binding during early neurogenesis. *Genome biology* 12, R2.
- 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.
- Matisse, M.P., and Wang, H. (2011). Sonic hedgehog signaling in the developing CNS where it has been and where it is going. *Current topics in developmental biology* 97, 75-117.

- McCaffery, P.J., Adams, J., Maden, M., and Rosa-Molinar, E. (2003). Too much of a good thing: retinoic acid as an endogenous regulator of neural differentiation and exogenous teratogen. *The European journal of neuroscience* 18, 457-472.
- McCarthy, R.A., Barth, J.L., Chintalapudi, M.R., Knaak, C., and Argraves, W.S. (2002). Megalin functions as an endocytic sonic hedgehog receptor. *The Journal of biological chemistry* 277, 25660-25667.
- Milon, B.C., Cheng, H., Tselebrovsky, M.V., Lavrov, S.A., Nenasheva, V.V., Mikhaleva, E.A., Shevelyov, Y.Y., and Nurminsky, D.I. (2012). Role of histone deacetylases in gene regulation at nuclear lamina. *PloS one* 7, e49692.
- Miyagi, S., Kato, H., and Okuda, A. (2009). Role of SoxB1 transcription factors in development. *Cell Mol Life Sci* 66, 3675-3684.
- Mizuguchi, R., Kriks, S., Cordes, R., Gossler, A., Ma, Q., and Goulding, M. (2006). *Ascl1* and *Gsh1/2* control inhibitory and excitatory cell fate in spinal sensory interneurons. *Nature neuroscience* 9, 770-778.
- Motoyama, J., Milenkovic, L., Iwama, M., Shikata, Y., Scott, M.P., and Hui, C.C. (2003). Differential requirement for *Gli2* and *Gli3* in ventral neural cell fate specification. *Developmental biology* 259, 150-161.
- 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.
- Mulieri, P.J., Okada, A., Sassoon, D.A., McConnell, S.K., and Krauss, R.S. (2000). Developmental expression pattern of the *cdo* gene. *Developmental dynamics : an official publication of the American Association of Anatomists* 219, 40-49.
- Novitsch, B.G., Wichterle, H., Jessell, T.M., and Sockanathan, S. (2003). A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. *Neuron* 40, 81-95.
- Nowling, T., Bernadt, C., Johnson, L., Desler, M., and Rizzino, A. (2003). The co-activator p300 associates physically with and can mediate the action of the distal enhancer of the *FGF-4* gene. *The Journal of biological chemistry* 278, 13696-13705.
- Nusslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* 287, 795-801.
- Pabst, O., Rummelies, J., Winter, B., and Arnold, H.H. (2003). Targeted disruption of the homeobox gene *Nkx2.9* reveals a role in development of the spinal accessory nerve. *Development* 130, 1193-1202.
- 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. *Molecular and cellular biology* 26, 3365-3377.
- Park, H.L., Bai, C., Platt, K.A., Matisse, 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.
- Patten, I., and Placzek, M. (2002). Opponent activities of Shh and BMP signaling during floor plate induction in vivo. *Curr Biol* 12, 47-52.
- Persson, M., Stamatakis, 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 & development* 16, 2865-2878.
- Pevny, L., and Placzek, M. (2005). SOX genes and neural progenitor identity. *Current opinion in neurobiology* 15, 7-13.
- Pierani, A., Brenner-Morton, S., Chiang, C., and Jessell, T.M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* 97, 903-915.
- Rhinn, M., and Dolle, P. (2012). Retinoic acid signalling during development. *Development* 139, 843-858.
- Ribes, V., Balaskas, N., Sasai, N., Cruz, C., Dessaud, E., Cayuso, J., Tozer, S., Yang, L.L., Novitsch, B., Marti, E., *et al.* (2010). Distinct Sonic Hedgehog signaling dynamics specify floor plate and ventral neuronal progenitors in the vertebrate neural tube. *Genes & development* 24, 1186-1200.

- Ribes, V., and Briscoe, J. (2009). Establishing and interpreting graded Sonic Hedgehog signaling during vertebrate neural tube patterning: the role of negative feedback. *Cold Spring Harbor perspectives in biology* 1, a002014.
- Robert, B. (2007). Bone morphogenetic protein signaling in limb outgrowth and patterning. *Development, growth & differentiation* 49, 455-468.
- Roeling, T.A., Docter, G.J., Voorn, P., Melchers, B.P., Wolters, E.C., and Groenewegen, H.J. (1995). Effects of unilateral 6-hydroxydopamine lesions on neuropeptide immunoreactivity in the basal ganglia of the common marmoset, *Callithrix jacchus*, a quantitative immunohistochemical analysis. *Journal of chemical neuroanatomy* 9, 155-164.
- 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.
- Rowitch, D.H., and Kriegstein, A.R. (2010). Developmental genetics of vertebrate glial-cell specification. *Nature* 468, 214-222.
- Ruiz i Altaba, A., Jessell, T.M., and Roelink, H. (1995). Restrictions to floor plate induction by hedgehog and winged-helix genes in the neural tube of frog embryos. *Mol Cell Neurosci* 6, 106-121.
- Sadler, T.W. (2005). Embryology of neural tube development. *American journal of medical genetics Part C, Seminars in medical genetics* 135C, 2-8.
- Sandberg, M., Kallstrom, M., and Muhr, J. (2005). Sox21 promotes the progression of vertebrate neurogenesis. *Nature neuroscience* 8, 995-1001.
- 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.
- Stamatakis, 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 & development* 19, 626-641.
- Stern, C.D. (2006). Neural induction: 10 years on since the 'default model'. *Current opinion in cell biology* 18, 692-697.
- te Welscher, P., Zuniga, A., Kuijper, S., Drenth, T., Goedemans, H.J., Meijlink, F., and Zeller, R. (2002). Progression of vertebrate limb development through SHH-mediated counteraction of GLI3. *Science* 298, 827-830.
- Tempe, D., Casas, M., Karaz, S., Blanchet-Tournier, M.F., and Concordet, J.P. (2006). Multisite protein kinase A and glycogen synthase kinase 3beta phosphorylation leads to Gli3 ubiquitination by SCFbetaTrCP. *Molecular and cellular biology* 26, 4316-4326.
- 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. *Developmental cell* 10, 647-656.
- Timmer, J.R., Wang, C., and Niswander, L. (2002). BMP signaling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription factors. *Development* 129, 2459-2472.
- Trompouki, E., Bowman, T.V., Lawton, L.N., Fan, Z.P., Wu, D.C., DiBiase, A., Martin, C.S., Cech, J.N., Sessa, A.K., Leblanc, J.L., *et al.* (2011). Lineage regulators direct BMP and Wnt pathways to cell-specific programs during differentiation and regeneration. *Cell* 147, 577-589.
- Ulloa, F., and Briscoe, J. (2007). Morphogens and the control of cell proliferation and patterning in the spinal cord. *Cell Cycle* 6, 2640-2649.
- van den Brink, G.R. (2007). Hedgehog signaling in development and homeostasis of the gastrointestinal tract. *Physiological reviews* 87, 1343-1375.
- Van Hateren, N., Belsham, A., Randall, V., and Borycki, A.G. (2005). Expression of avian Groucho-related genes (Grgs) during embryonic development. *Gene Expr Patterns* 5, 817-823.

- VanderMeer, J.E., and Ahituv, N. (2011). cis-regulatory mutations are a genetic cause of human limb malformations. *Developmental dynamics : an official publication of the American Association of Anatomists* 240, 920-930.
- Visel, A., Blow, M.J., Li, Z., Zhang, T., Akiyama, J.A., Holt, A., Plajzer-Frick, I., Shoukry, M., Wright, C., Chen, F., *et al.* (2009). ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854-858.
- 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.
- Vokes, S.A., Ji, H., Wong, W.H., and McMahon, A.P. (2008). A genome-scale analysis of the cis-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb. *Genes & development* 22, 2651-2663.
- Ward, L.D., and Kellis, M. (2012). Interpreting noncoding genetic variation in complex traits and human disease. *Nat Biotechnol* 30, 1095-1106.
- 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 & development* 16, 2849-2864.
- Williams, R.W., and Herrup, K. (1988). The control of neuron number. *Annual review of neuroscience* 11, 423-453.
- Winterbottom, E.F., Illes, J.C., Faas, L., and Isaacs, H.V. (2010). Conserved and novel roles for the Gsh2 transcription factor in primary neurogenesis. *Development* 137, 2623-2631.
- Winterbottom, E.F., Ramsbottom, S.A., and Isaacs, H.V. (2011). Gsx transcription factors repress Iroquois gene expression. *Developmental dynamics : an official publication of the American Association of Anatomists* 240, 1422-1429.
- Xie, J., Murone, M., Luoh, S.M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J.M., Lam, C.W., Hynes, M., Goddard, A., *et al.* (1998). Activating Smoothed mutations in sporadic basal-cell carcinoma. *Nature* 391, 90-92.
- Zhan, X., Shi, X., Zhang, Z., Chen, Y., and Wu, J.I. (2011). Dual role of Brg chromatin remodeling factor in Sonic hedgehog signaling during neural development. *Proceedings of the National Academy of Sciences of the United States of America* 108, 12758-12763.
- Zhang, X., Heaney, S., and Maas, R.L. (2003). Cre-loxp fate-mapping of Pax6 enhancer active retinal and pancreatic progenitors. *Genesis* 35, 22-30.
- Zhang, X.M., Ramalho-Santos, M., and McMahon, A.P. (2001). Smoothed mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. *Cell* 105, 781-792.
- Zhao, L., and Koopman, P. (2012). SRY protein function in sex determination: thinking outside the box. *Chromosome Res* 20, 153-162.
- Zhou, Z.D., Kumari, U., Xiao, Z.C., and Tan, E.K. (2010). Notch as a molecular switch in neural stem cells. *IUBMB life* 62, spcone.