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**Authors**

Andrews, Madeline G  
Kong, Jennifer  
Novitch, Bennett G  
et al.

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**New perspectives on the mechanisms establishing the dorsal-ventral axis of the spinal cord**

**Madeline G. Andrews<sup>1,2</sup>, Jennifer Kong<sup>1,2</sup>, Bennett G. Novitch<sup>1,3</sup>, and Samantha J. Butler<sup>1,3</sup>**

1. Department of Neurobiology,
2. Neuroscience Graduate Program,
3. Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research,  
University of California, Los Angeles  
Los Angeles, CA 90095

\* Correspondence: [butlersj@ucla.edu](mailto:butlersj@ucla.edu)

310 206 8416

TLSB 3129, 610 Charles E Young Drive East,  
University of California, Los Angeles,  
Los Angeles CA 90095

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## **Abstract**

Distinct classes of neurons arise at different positions along the dorsal–ventral axis of the spinal cord leading to spinal neurons being segregated along this axis according to their physiological properties and functions. Thus, the neurons associated with motor control are generally located in, or adjacent to, the ventral horn whereas the interneurons (INs) that mediate sensory activities are present within the dorsal horn. Here, we review classic and recent studies examining the developmental mechanisms that establish the dorsal-ventral axis in the embryonic spinal cord. Intriguingly, while the cellular organization of the dorsal and ventral halves of the spinal cord look superficially similar during early development, the underlying molecular mechanisms that establish dorsal vs. ventral patterning are markedly distinct. For example, the ventral spinal cord is patterned by the actions of a single growth factor, sonic hedgehog (Shh) acting as a morphogen, i.e. concentration-dependent signal. Recent studies have shed light on the mechanisms by which the spatial and temporal gradient of Shh is transduced by cells to elicit the generation of different classes of ventral INs, and motor neurons (MNs). In contrast, the dorsal spinal cord is patterned by the action of multiple factors, most notably by members of the bone morphogenetic protein (BMP) and Wnt families. While less is known about dorsal patterning, recent studies have suggested that the BMPs do not act as morphogens to specify dorsal IN identities as previously proposed, rather each BMP has signal specific activities. Finally, we consider the promise that elucidation of these mechanisms holds for neural repair.

## **I. Functional organization of the adult spinal cord**

The central nervous system (CNS) is comprised of the brain and spinal cord, and is collectively responsible for much of what we consider to be vital for life and the experience of being human: cognition, movement, emotion, sensation, respiration and learning. While the spinal cord is less structurally complex than the brain, it nonetheless performs an essential task, receiving and processing incoming sensory information from the peripheral nervous system and then modulating and/or executing appropriate motor outputs (Andersson et al., 2012; Goetz et al., 2015; Lai et al., 2016) (Fig. 1A). The spinal cord coordinates movement, orients our bodies in space, quickly processes harmful stimuli, and allows us to respond to our environment. The cells that process these sensory and motor functions are organized along the dorsal-ventral axis. Motor neurons (MNs) in the ventral horn control and coordinate motor output. Motor circuits are comprised of MNs, whose axons exit the spinal cord through the ventral root to synapse onto specific muscles, and spinal interneurons (INs), which modulate the activity of MNs. In contrast, sensory circuits relay sensory information from the periphery to the brain and motor systems. Different populations of peripheral sensory neurons in the dorsal root ganglion (DRG) detect distinct types of sensory information and send afferent projections either directly onto MNs or into the dorsal horn of spinal cord, where they synapse onto spinal INs. These dorsal INs then process and relay the relevant categories of sensory information either to the brain (Fig. 1B) or to MNs to modulate reflex-specific MN output (Arber, 2012).

In the adult spinal cord, the dorsal horn is organized with a layered, or laminar, architecture (Rexed, 1954). Seven laminae contain neurons with distinct physiological properties; some neurons locally process afferent sensory information while others relay it to higher order centers in the brain including the brainstem, thalamus and cerebellum (Bermingham et al., 2001) (Brown and Fyffe, 1981; Lai et al., 2016; Todd, 2010). Information about nociception (pain) (Villeda et al., 2006; Xu et al., 2013; Yasaka et al., 2010) or thermosensation (temperature) is processed in layers I-II, the most superficial layers of the horn (Bautista et al., 2007; Szabo et al., 2015). Somatosensation (touch) is processed in the intermediate layers, III-IV (Bourane et al., 2015b), and finally, proprioception, or perception of where your body resides in space, is processed in the deep horn, layers V-VI (Miesegaes et al., 2009; Yang et al., 2001; Yuengert et al., 2015) (Fig. 1B). While the type of information processed in each laminae is generally understood, the transcriptional identities

and functional characteristics of the IN subpopulations present in each laminae are still being characterized (Abraira et al., 2017; Bourane et al., 2015a; Bourane et al., 2015b).

In the ventral horn of the spinal cord (layer IX), MNs are segregated into distinct clusters, called pools, which innervate individual or groups of muscles. Motor pools are topographically arranged according to their muscle targets. They form columns along the rostral-caudal axis, settling at the appropriate axial position to innervate adjacent muscle targets (Dasen et al., 2008; Jessell, 2000; Jessell et al., 2011; Rousso et al., 2008). The most medial MNs become the medial motor column (MMC), which innervates back and vertebral muscles. In the cervical and thoracic spinal cord, the hypaxial motor column (HMC) lies slightly medial to the MMC and innervates trunk muscles, many of which are associated with respiration. The thoracic spinal cord also contains the more laterally positioned preganglionic motor column (PGC) MNs which innervate sympathetic targets. At forelimb and hindlimb levels, are two lateral motor columns (LMC) which are divided into medial and lateral sub-columns, LMC<sub>m</sub> and LMC<sub>l</sub>, to respectively control flexor and extensor muscles. The remaining layers of the ventral horn contain a multitude of interneurons that coordinate the activities of MNs (Goulding, 2009).

### **The organization of the embryonic spinal cord along the dorsal-ventral axis**

Over the past 30 years, the embryonic spinal cord has emerged as an exceptional model system for assessing the mechanisms that direct the specification of neuronal and glial cell fate. It has a relatively simple architecture, arising when the neural plate folds to form the neural tube from a pseudostratified epithelium, which is only one cell thick at the earliest stages (Fig. 2A). The neural tube is comprised of rapidly dividing cells, which are the progenitors for all of the neurons and glia in the spinal cord. As the progenitors differentiate, the cells migrate laterally such that a simple laminar structure is formed: the inner ventricular zone contains the nuclei of the proliferating progenitors; the mantle layer contains the post-mitotic neural cell bodies; and the outer marginal layer is comprised of processes. At the earliest stages, the marginal layer contains the thin processes of the neuroepithelial cells; however, it expands over time to accommodate the fiber tracts, becoming better known as the white matter of the adult spinal cord. The mantle layer ultimately becomes the grey matter.

The identities of the progenitors within the ventricular zone are patterned by inductive signaling molecules released from adjacent structures. These signals include the bone morphogenetic proteins (BMPs) and Wnt family from the roof plate (RP) at the dorsal midline, sonic hedgehog (Shh) from the floor plate (FP) at the ventral midline, and retinoic acid (RA) from somites in the adjacent paraxial mesoderm (Briscoe and Novitch, 2008; Tanabe and Jessell, 1996) (Fig. 2B). These signals compete and cooperate to specify distinct domains of progenitors along the dorsal-ventral axis. Domains of progenitors become distinct from each other following the expression of unique complements of transcription factors specifying their identities. Upon neurogenesis, patterned progenitor cells exit the cell cycle and migrate laterally into the mantle layer (Helms and Johnson, 2003; Le Dreau and Marti, 2013) (Fig. 2B-C). This process in turn results in discrete populations of neurons arrayed along the dorsal-ventral axis at the lateral edge of the spinal cord. The dorsal spinal cord contains at least six dorsal progenitor (dP) domains, dP1-dP6, that differentiate into the dorsal interneuron (dI) populations 1-6 (Fig. 2C) (Helms and Johnson, 2003). Each neuronal population is distinguished by its expression of a distinct subset of transcription factors which appear to regulate their position, development and later connectivity and function (Lai et al., 2016). The dIs mature into sensory relay INs. The function of many of these neural populations have only recently been identified. The dI1s mediate proprioception (Bermingham et al., 2001; Yuengert et al., 2015), the dI3s facilitate some mechanosensory behaviors including grasping control (Bui et al., 2013; Goetz et al., 2015), the dI4s and dI5s have multiple functions including itch/touch/pain (Abraira et al., 2017; Bourane et al., 2015a; Bourane et al., 2015b) and the dI6s regulate motor output (Lai et al., 2016). (Fig. 1).

The ventral spinal cord encompasses at least five progenitor (p) domains, p0-p3 and the pMN, which give rise to five distinct cell types, the ventral (V) IN, V0-V3 and MNs (Briscoe and Novitch, 2008; Goulding, 2009; Jessell, 2000). The MNs control movement throughout the body axis, while the ventral INs, V0-V3, function to modulate motor activities. The V0-V3 IN populations are a mix of ipsilaterally and contralaterally projecting populations that regulate MN output (Gosgnach et al., 2017). These populations are further subdivided based on their dorsal-ventral position, axon projection type and function. Broadly, V0 and V2 are a mix of excitatory and inhibitory INs, while V1s are inhibitory and V3 are excitatory (Gosgnach et al., 2017;

Goulding, 2009). V0 and V2 neurons serve to alternate left/right motor activities (Crone et al., 2008; Griener et al., 2015) while V1 and V2 coordinate flexor-extensor activities (Zhang et al., 2014). V1 neurons also influence motor speed (Gosgnach et al., 2006), while V3 modulate the quality of motor bursts as well as overall motor performance (Zhang et al., 2008). As with the dorsal neural populations, considerable work has defined the transcription factor code that specify the identity of the motor pools, and ventral INs (Catela et al., 2015; Gosgnach et al., 2017).

## **II. The specification of dorsal and ventral patterning in the spinal cord**

The cellular mechanisms that establish the embryonic dorsal and ventral spinal cord are remarkably similar. Many of the neural populations are specified by inductive signaling from clusters of glial-like cells at the opposing ventral and dorsal midlines. These cells have distinct origins: the dorsal RP is formed in the region where the neural plate seals to form the neural tube, whereas the ventral FP is induced by signals from the underlying notochord, a transient mesodermal structure (Yamada et al., 1991). These structures are considered to be analogous signaling centers, secreting inductive factors that pattern the identity of the adjacent tissue. However, the identity of these factors, and the mechanisms used to pattern cell fate have recently been shown to be strikingly different. We will begin by reviewing the mechanisms of ventral patterning, which has been extensively studied as a model for morphogen signaling, and then discuss how the mechanisms of dorsal patterning appear to use a signal specific strategy.

### **The Hh signaling pathway**

In vertebrates, the hedgehog (Hh) signaling pathway is activated when extracellular Hh ligands bind to the transmembrane receptor patched (Ptch) (Chen and Struhl, 1996; Ingham et al., 1991; Marigo et al., 1996; Stone et al., 1996). This interaction relieves Ptch-mediated inhibition of the transmembrane protein Smo, allowing for the activation and translocation of Smo into the primary cilium, a single nonmotile microtubule-based projection present on nearly all vertebrate cells (Corbit et al., 2005; Rohatgi et al., 2007). The presence of activated Smo within the primary cilium triggers a cascade of downstream signaling events that culminates in the conversion of Gli proteins into transcriptional activators (GliA) that translocate into the nucleus and directly turn on downstream genes including *Gli1* and *Ptch* (Goodrich et al., 1996; Lee et al., 1997) (Fig, 3B). In the absence of Hh ligands, Ptch represses both the activation and ciliary localization of Smo,

resulting in the truncation of Gli proteins into transcriptional repressors (GliR) (Hui et al., 1994; Hynes et al., 1997; Ruiz i Altaba, 1998; Sasaki et al., 1999) (Fig. 3A). While the link between Smo activation and Gli processing has been well established, the mechanistic details explaining this connection remain unclear.

Due to genome duplication events in early vertebrates (Wada and Makabe, 2006) there are three mammalian Hh genes: *sonic hedgehog* (*Shh*); *indian hedgehog* (*Ihh*); and *desert hedgehog* (*Dhh*) (Hammerschmidt et al., 1997). The three mammalian Hh proteins are remarkably similar, as they are all processed by the same mechanisms, have the capacity to bind to the Hh receptors patched1 and patched2 (Ptch1 and Ptch2), and ultimately upregulate the expression of known Hh pathway target genes (Carpenter et al., 1998). However, each Hh protein has a unique expression pattern in the developing embryo. Shh is the most broadly expressed member of the mammalian Hh family and is the key signal that patterns the ventral spinal cord (Echelard et al., 1993; Varjosalo and Taipale, 2008).

### **Shh acts as a canonical morphogen in ventral patterning**

Ventral patterning commences in the spinal cord when signals from the notochord induce the formation of the FP. This inductive interaction was first observed by Johannes Holtfreter in the 1930s (Gerhart, 1996), but the molecule responsible was not identified until many decades later. It took an unexpected convergence of molecular and evolutionary genetics in the late 1980s for researchers to realize that the genes responsible for patterning segments in *Drosophila* (Lee et al., 1992; Mohler and Vani, 1992; Nusslein-Volhard and Wieschaus, 1980; Tabata et al., 1992; Tashiro et al., 1993) were also critically involved in the organization of cellular identity in the vertebrate embryo.

Ventral patterning was found to be induced by a single signaling molecule, Shh, identified by its homology with the *Drosophila* gene, hedgehog (Roelink et al., 1994). Shh is secreted from the notochord and induces the formation of the FP at the ventral midline (Roelink et al., 1994). The FP itself then also secretes Shh, which specifies neuronal identity by acting as a morphogen (Ericson et al., 1997a; Yamada et al., 1993) (Fig. 2A). Morphogens were first hypothesized to exist as diffusible factors that act in a concentration-dependent manner to direct cellular identity



(Bier and De Robertis, 2015; Briscoe et al., 1999; Wolpert, 1969). Shh is one of relatively few molecules that have been credibly shown to act as a canonical morphogen during development. In a classic series of experiments, Thomas Jessell and co-workers used spinal tissue culture assays to demonstrate that high concentrations of solubilized Shh specify the ventral-most identities in the spinal cord, FP and p3, while progressively lower concentrations specify more intermediate cell types, i.e. the pMN, and then V2-V0 (Ericson et al., 1997a; Roelink et al., 1995). Subsequent analyses showed that loss of Shh *in vivo* resulted in the profound loss of ventral spinal identities, far beyond the FP, the source of *Shh* expression (Chiang et al., 1996). These experiments are among the some of the most convincing to demonstrate that morphogens exist in vertebrates, suggesting a model whereby a localized, diffusible signal at the FP patterns the adjacent tissue into discrete domains of specific neuronal identities (Tanabe and Jessell, 1996). The morphogen model of patterning has been highly influential, with other localized sources of inductive signals being hypothesized to pattern other areas of the CNS, including the dorsal spinal cord (Liem et al., 1997).

### **The Shh gradient is interpreted by a code of Gli transcription factors**

The Hh signal transduction pathway converges on Gli proteins, an evolutionarily conserved family of zinc finger transcription factors. Vertebrates have three Gli homologs: Gli1, Gli2, and Gli3. Gli1 only exists as a transcriptional activator and primarily serves as a secondary amplifier of Hh signaling activity (Bai et al., 2002; Lee et al., 1997). In contrast, Gli2 and Gli3 can become either transcriptional activators or repressors because they possess both a C-terminal activator domain and a N-terminal repressor domain. Despite this bifunctional potential, Gli2 is typically processed into a transcriptional activator and Gli3 into a repressor through differential phosphorylation of the proteins at specific sites that modulate transcriptional activities as well as proteolytic cleavage of their C-terminal activator domains (Aza-Blanc et al., 2000; Hui and Angers, 2011; Niewiadomski et al., 2014; Persson et al., 2002; Ruiz i Altaba, 1998; Sasaki et al., 1999).

Within the developing spinal cord, Shh is initially synthesized and secreted from the notochord and later the FP of the neural tube (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994). Over time, Shh diffuses dorsally away from this fixed source, generating a high-to-low Shh concentration gradient along the ventral-to-dorsal axis of the neural tube (Burke et al., 1999; Chamberlain et al., 2008; Goetz et al., 2006; Zeng et al., 2001) (Fig. 3C). This extracellular Shh

concentration gradient is translated into an intracellular gradient of Gli activity, whereby GliA levels are elevated in the ventral spinal cord and GliR is elevated in the dorsal spinal cord (Fig. 3C). The balance between GliA and GliR, defined as net Gli activity, ultimately regulates the expression of transcription factors that will define the progenitor identity of the cells (Stamatakis et al., 2005). Within the spinal cord, the transcription factors regulated by net Gli activity are divided into two classes: the dorsally expressed class I proteins (Dbx2, Dbx1, Pax6, and Irx3), which are repressed by Shh signaling activity, and the ventrally expressed class II proteins (Nkx6.1, Nkx6.2, Olig2, and Nkx2.2) that are activated by Shh signaling (Briscoe et al., 2000; Ericson et al., 1997b). The dorsal/ventral limits of transcription factor expression are a consequence of Shh signaling activity, as the well-defined boundaries between progenitor domains are established by cross repressive interactions between complementary class I/II protein pairs. Ultimately, unique combinations of class I and class II proteins subdivide the ventral neural tube into five molecularly distinct progenitor domains (p3, pMN, p2, p1, and p0) (Fig. 3C). Each of these progenitor domains subsequently gives rise to a distinct subset of neurons (V3, MN, V2, V1, and V0) and glial cells later in development (Rowitch and Kriegstein, 2010).

### **Shh also functions as a temporal morphogen**

In addition to the specific extracellular concentration of Shh, the transcriptional profile of a ventral neural progenitor cell is dependent on the duration of time it is exposed to Shh. This temporal component of Shh signaling is evident in the early embryonic spinal cord, where ventral progenitor domains gradually appear over time as a result of prolonged Shh signaling activity driving the sequential expression of increasingly ventral class II transcription factors (Dessaud et al., 2010; Dessaud et al., 2007). Remarkably, this progressive patterning process can be reproduced *in vitro*, by exposing chick neural tube explants and mouse neural progenitor cells to Shh ligands and Smo agonists for extended durations of time (Dessaud et al., 2010; Dessaud et al., 2007; Kutejova et al., 2016; Sagner et al., 2018).

When Shh is absent from these cultures, neural progenitors express class I transcription factors, such as Pax6 and Pax7. The addition of Shh turns on the class II transcription factors. Initially, Nkx6.1 and Olig2 (markers of p2 and pMN state) are induced by low levels of Shh signaling activity and reductions in GliR levels. However, sustained exposure to Shh over time results in

the accumulation of GliA and expression of the p3-associated transcription factor Nkx2.2, which itself serves as a repressor of Olig2 expression and pMN fate. This shift in patterning occurs due to temporal adaptation to the morphogen. Prolonged exposure to Shh causes progenitor cells to upregulate Ptch, which functions as a negative feedback inhibitor of the Shh signaling pathway. Increased Ptch levels cause progenitors to become insensitive to extracellular Shh over time (Balaskas et al., 2012; Dessaud et al., 2010; Dessaud et al., 2007; Jeong and McMahon, 2005). Only the ventral-most progenitor cells, exposed to the highest concentrations of extracellular Shh, maintain the elevated levels of Shh signaling activity needed to turn on the transcription factors such as Nkx2.2 that define the ventral-most domains.

While exposure to Shh causes progenitors to become desensitized to Shh, it was recently shown that Notch signaling can sustain a progenitor's responsiveness to Shh over time (Kong et al., 2015; Stasiulewicz et al., 2015). Experiments using both transgenic mice and neural progenitor cultures demonstrated that elevating Notch signaling activity extends the duration of time a cell is maintained as a Shh-responsive progenitor. This increase in Shh signaling activity shifts the fate of the progenitors to a more ventral identity. Interactions between Notch and Shh signaling have been described in other systems (Casso et al., 2011; Hallahan et al., 2004; Huang et al., 2012; Lopez et al., 2003). Shh pathway components are directly regulated by the Rbpj transcriptional complex (Hamidi et al., 2011; Li et al., 2012), and elevated Notch signaling promotes the Shh driven accumulation of Smo to the primary cilia (Kong et al., 2015). However, the precise means through which Notch signaling interacts with Shh signaling remains largely unknown.

### **The RA signaling pathway**

Retinoic acid (RA) signaling is a core developmental signaling pathway that also contributes to dorsal-ventral patterning in the spinal cord. Derived from dietary vitamin A, the importance of RA in development is perhaps best illustrated from studies on vitamin A deficiencies. When pregnant female rats were maintained on a vitamin A-deficient diet over a short gestational window, the resulting pups exhibited wide spread developmental defects to the eyes, skull, trachea, esophagus, limbs, heart, and CNS (Dickman et al., 1997).

When vitamin A (all-trans retinol, at-retinol) is consumed, it is absorbed into the blood stream via

the small intestine, where it binds to retinol binding protein 4 (RBP4). RBP4 serves as a tag, binding to the membrane receptor STRA6 and allowing for at-retinol to identify and enter the cytoplasm of target cells. Once in the cytoplasm, at-retinol is converted into its biologically active form, all-trans retinoic acid (RA), via a two-step oxidation process involving the enzymes retinol or alcohol dehydrogenases (RoDHs or ADHs) and retinaldehyde dehydrogenases (RALDHs) (Maden, 2002). RA is then shuttled into the nucleus by binding to cellular retinoic acid binding protein 2 (CRABP2) where it binds to either the RA receptors (RARs) or the retinoid X receptors (RXRs). These RA bound receptors (RA-RAR and RA-RXR) then heterodimerize and bind to retinoic-acid response elements (RAREs) on target genes to activate their transcription (Kam et al., 2012; Maden, 2007). Through the use of ChIP-on-chip (chromatin immunoprecipitation combined with microarray hybridization) and ChIP-seq technology, over 300 potential RA-regulated genes have been identified (Delacroix et al., 2010). Among the targets were Hox genes and components of the TGF $\beta$  (transforming growth factor  $\beta$ ) signaling pathway, a pathway known to interact with RA signaling to regulate cell growth and differentiation.

In the developing CNS, RA signaling plays a major role in both anteroposterior and dorsal-ventral progenitor patterning. Multiple studies have shown that RA signaling drives progenitor cells towards more posterior/rostral cell identities by directly binding to and manipulating the expression of certain Hox genes (Conlon and Rossant, 1992; Delacroix et al., 2010; Durston et al., 1989; Huang et al., 2002; Koop et al., 2010). Additional studies conducted in mouse embryonic cell culture, avian embryos, and chick neural plate explants have also shown that RA drives spinal cord progenitors towards a more dorsal identity (Novitch et al., 2003; Okada et al., 2004; Wilson et al., 2004). One of the ways in which RA contributes to fate specification is by positively regulating the expression of class I transcription factors, thereby opposing the ventral patterning actions of the Shh pathway (Briscoe and Novitch, 2008; Novitch et al., 2003). Beyond its role in patterning, RA signaling can also drive progenitors towards neuronal differentiation (Sharpe and Goldstone, 1997).

### **The specification of dorsal patterning in the dorsal spinal cord**

The similarity between the organization of early dorsal and ventral spinal cord led to the hypothesis that the molecular mechanisms that assembled cellular identity were also similar. However, while

the ventral spinal cord is patterned largely by the action of one molecule, multiple members of the BMP and Wnt families are critical for dorsal patterning. It was thus unclear how >5 growth factors could cooperate to form a morphogen gradient. Indeed, recent studies have found no evidence that the BMPs act as morphogens to pattern the spinal cord, rather each BMP ligand directs a unique range of dorsal cellular identities.

### **The BMP signaling pathway**

There are ~30 BMP family members expressed during embryonic development (Ducy and Karsenty, 2000). Although they were first discovered by their ability to direct the formation of bone and cartilage (Urist et al., 1968), they are also required for the development of the dorsal spinal cord, limb, eye, forebrain and the kidney (Ahn et al., 2001; Dudley et al., 1995; Lyons et al., 1995; Wyatt et al., 2010). BMPs can regulate many processes including cell division, apoptosis, proliferation, cell migration, cell survival and differentiation (Bier and De Robertis, 2015; Ducy and Karsenty, 2000; Hogan, 1996; Shi and Massague, 2003; Wu and Hill, 2009). The BMPs signal through a complex of type I and type II serine/threonine kinase receptors (Balemans and Van Hul, 2002) (Fig. 4A). Signal specificity is thought to reside in the identity of the BMP type I receptor (BmprI), which include the BmprIa (also known as Alk3), and BmprIb (Alk6) (Moustakas and Heldin, 2009; ten Dijke et al., 1994). On BMP binding, BmprII transphosphorylates BmprI, which in turn phosphorylates and activates the Smad proteins, the intracellular mediators of canonical BMP signaling (Shi and Massague, 2003) (Fig 4A).

There are three known classes of Smads: the receptor (R) activated, inhibitory (I) and common mediator (Co) Smads (Balemans and Van Hul, 2002). On phosphorylation by BmprI, the R-Smads complex with Co-Smad4 and translocate into the nucleus to regulate transcription. (Moustakas and Heldin, 2009). Three of the R-Smads, Smad1, Smad5 and Smad8, are considered BMP specific, while two, Smad2 and Smad3, are activated by the TGF $\beta$  pathway. The I-Smads, Smad6 and Smad7, are involved in the negative regulation of BMP signaling (Fig. 3) (Massague et al., 2005; Wu and Hill, 2009). Smad6 competitively binds to phosphorylated R-Smads to inhibit the formation of the complex with Co-Smad4 (Fig 4A). Smad7 competes with the R-Smads for binding to activated BmprIs. Additionally, both I-Smads, Smad6 and Smad7, can regulate BMP

receptor degradation by promoting ubiquitination in conjunction with another inhibitory molecule, Smufl (Massague et al., 2005).

### **BMPs were first proposed to signal as a morphogens to pattern the dorsal spinal cord**

During neurulation, BMPs in the non-neural ectoderm promote the formation of the RP at the dorsal midline of the spinal cord (Liem et al., 1995). Subsequently, the RP itself expresses a range of BMPs in nested domains (Butler and Dodd, 2003; Lee et al., 1998; Liem et al., 1997). The rodent RP expresses at least three members of the BMP family, BMP6, BMP7 and Growth/Differentiation Factor (GDF) 7. The complement of BMPs expressed in the chicken RP is larger, comprising at least four members of the BMP family, BMP4, BMP5, BMP7 and BMP9 (dorsalin1) (Liem et al., 1997). At later stages, the expression of the BMPs extends more broadly into the dorsal spinal cord (Andrews et al., 2017; Le Dreau and Marti, 2013). Studies examining the loss (Hazen et al., 2012; Le Dreau et al., 2012; Lee et al., 1998; Nguyen et al., 2000; Wine-Lee et al., 2004) or gain (Andrews et al., 2017; Chizhikov and Millen, 2004; Timmer et al., 2005; Timmer et al., 2002; Yamauchi et al., 2008) of BMP signaling have shown that BMP signaling is critical *in vivo* for the formation of the dI1, dI2 and dI3 classes of sensory neurons. The I-Smads, Smad6 and Smad7, appear to act to confine the activities of the BMPs to the dorsal-most spinal cord (Hazen et al., 2011).

The mechanism by which the BMPs promote dorsal cell fate had remained unresolved until recently. The BMPs were first proposed to act collectively to establish a morphogen gradient (Lee and Jessell, 1999), by analogy with the Shh morphogen model in the ventral spinal cord (Ericson et al., 1997b; Yamada et al., 1993). In the canonical spatial morphogen model, high levels of BMP signaling should induce the dorsal-most fates, i.e. the RP and dI1s, whereas lower levels of BMPs should induce more ventral dorsal cell types, such as dI3s. Studies in the Jessell laboratory demonstrated that conditioned medium from either BMP4- or GDF7-transfected COS cells was sufficient to specify dI1s in naïve chicken neural plate tissue explants (Lee et al., 1998; Liem et al., 1997). However, treatment of these explants with diluted BMP4-conditioned medium was not

particularly effective at inducing dl3s, the predicted result if BMP4 functioned as a canonical spatial morphogen (Liem et al., 1997).

A more recent study has suggested that BMP4 functions as a “temporal” morphogen (Tozer et al., 2013), again by analogy with Shh signaling (Dessaud et al., 2007). Dorsal progenitor identities within chicken neural plate explants were observed to change when cultured with BMP4 for different durations of time. The explants were first directed toward the dP2 identity, but then assumed the more dorsal dP1 fate as time progressed, suggesting that progenitors can integrate BMP signaling over time to assume a more dorsal spinal identity. Collectively, these experiments provided some evidence that there is a concentration- and/or timing-dependent component to activity of the BMPs. However, none of these studies addressed a key difference between signaling in the RP and FP: how the multiple members of the BMP family present in the dorsal spinal cord, might cooperate to establish a unified morphogen gradient.

### **BMPs have distinct roles directing dorsal spinal fates**

An alternative model is that the different BMPs have unique activities specifying the induction of particular neural fates (Lee and Jessell, 1999). Early support for this hypothesis came when loss of the *Gdf7* gene in mice was found to specifically ablate a subpopulation of dI1s without affecting the identity of the other populations of dorsal INs (Lee et al., 1998). More recently, knocking down the expression of *Bmp4* and *Bmp7* in chicken embryos was shown to have qualitatively different effects: decreasing *Bmp4* expression reduces the number of dI1s, while depleting *Bmp7* expression reduces the number of dI1s, dI3s and dI5s (Le Dreau and Marti, 2013). These findings support the hypothesis that BMP ligands have unique activities specifying dorsal cell fates.

We have recently resolved the mode by which the different BMP ligands direct dorsal spinal identity. The ability of RP-derived BMPs to direct dorsal spinal fates was methodically tested by either manipulating *Bmp* expression in chicken embryos *in vivo* (Andrews et al., 2017), or culturing mouse (m) or human (h) embryonic stem cells (ESC) with different BMP recombinant proteins *in vitro* (Andrews et al., 2017; Gupta et al., 2018). These studies demonstrated unambiguously that BMPs do not act as concentration-dependent morphogens. Dose-response experiments in both the chicken and mouse systems showed that while altering the level of BMPs changed the

effectiveness of the response, i.e. the number of cells converted to a specific fate, there were none of the changes in the dorsal IN identity predicted by either the spatial or temporal morphogen models.

Rather, BMPs were observed to be distinct in their abilities to direct the four dorsal-most cell types: the RP, dI1, dI2 and dI3. Each BMP has a complex range of activities, with some species-specific variation: they have overlapping and distinct functions directing progenitor patterning and the specification of dorsal IN identities. Thus, BMP6 (mouse) and BMP7 (chicken) are the most effective at directing the RP fates (Fig. 4B). BMP4 and BMP7 both promote dP1 proliferation (chicken), but only BMP4 can direct progenitors to differentiate as dI1s (mouse and chicken) (Fig. 4C). BMP4 is the only BMP that can direct dP2 to differentiate into dI2s in chicken (Fig. 4D). All BMPs tested in both species, including BMP4, BMP5, BMP6 and BMP7, can promote modest levels of dP3 proliferation and their differentiation into dI3s (Fig. 4E). No BMP was identified that direct the dI4-dI6 fates, rather BMP signaling tends to suppress the ventral-dorsal fates, consistent with earlier findings (Hazen et al., 2011). Thus, the BMPs have an evolutionarily conserved role specifying the dorsal spinal cord, with a species-specific code of BMP signals that directs dorsal IN formation.

BMPs mediate their signal-specific activities patterning the dorsal spinal cord by activating different type I Bmprs. In both chicken and mouse, the activities of BMP4 are mediated through *Bmpr1b*. In contrast, the activities of BMP7 (chicken) and BMP6 (mouse) are mediated, at least in part, by *Bmpr1a* (Andrews et al., 2017). However, the mechanisms by which different BMP receptor complexes then promote distinct dorsal cell identities remain unclear. Previous studies have differed in their conclusions as to whether both Smad1 and Smad5 (Le Dreau et al., 2012) or Smad5 alone (Hazen et al., 2012) mediate the specification of dorsal IN fate. R-Smads (Smad1/5/8) are phosphorylated (p), i.e. activated, in a gradient in the dorsal-most spinal cord (Hazen et al., 2012). This distribution suggested that specific levels of Smad activation might encode specific cell fates. However, this hypothesis has not been supported by recent studies: misexpression of *Bmp4* and *Bmp7* can direct similar levels of pSmad, yet results in different cell fate outcomes (Andrews et al., 2017). Thus, it appears that the R-Smads can be activated in a BMP-specific manner to result in distinct patterning activities. Future studies need to evaluate



how the type I Bmprs differentially regulate the ability the R-Smads to turn on the specific genes involved the assumption of specific dorsal cell fates.

### **The specification of dorsal spinal fate may be discontinuous**

A curious, and mechanistically unresolved, feature of patterning along the dorsal spinal cord is the apparently discontinuous specification of dorsal IN identity, akin the expression of pair rule genes in the specification of segments in the *Drosophila* embryo (Pankratz and Jäckle, 1990). This finding was first reported by Elisa Martí and co-workers when they reported that BMP7 signaling is required for the generation of dI1, dI3 and dI5 subpopulations (Le Dreau et al., 2012). They found the depletion of *Bmp7* expression reduces the number of dI1, dI3 and dI5 by ~50% in chicken embryos using RNA knockdown approaches, and by ~25% in *Bmp7* mutant mouse embryos. However, the identity of the BMP mediating these activities remains unclear, as this phenotype was not previously observed in *Bmp7* mutants (Butler and Dodd, 2003), and gain of function studies in mESC cultures using BMP7 recombinant protein do not have the same effects on dorsal IN identity (Andrews et al., 2017). However, the ability of individual BMPs to induce discreet populations of dorsal INs is consistent across these studies. BMP4 is sufficient to specify dI1 and dI3 both *in vivo* and *in vitro*. *Bmp4* misexpression does not induce additional dI5s in chicken embryos *in vivo*, but its effect on dI5s has not yet been assessed in mESC or hESC cultures *in vitro*.

Moreover, the dI2, dI4 and dI6 subpopulations may be formed in a similarly discreet manner. BMP signaling is both necessary (Wine-Lee et al., 2004) and sufficient (Andrews et al., 2017) for the differentiation of dI2s *in vivo*. Recent chicken misexpression studies have shown that *Bmp4* elevation drives the *Ngn1*<sup>+</sup> domain of dP2s to rapidly differentiate into dI2s, thereby depleting the dP2 population (Fig. 4D). While low levels of *Bmp4* expression solely deplete dP2, higher *Bmp4* expression levels can additionally deplete a more ventral *Ngn1*<sup>+</sup> domain, which includes dP6, resulting in the formation of a large mass of dI2-like cells (Andrews et al., 2017). Unexpectedly, we were unable to derive BMP-dependent dI2s *in vitro* in either mESC or hESC cultures. However, these cultures are also treated with RA, to drive the ESCs toward a spinal neural identity (Wichterle et al., 2002). dI2s and neurons with shared dI4/dI6 identities were rather found to be

arising in the RA-alone “control” condition in the hESC directed differentiation protocol (Gupta et al., 2018).

These disparate findings are complex to interpret but are generally consistent with a model in which the dorsal progenitor domains are first subdivided into either a dP1/dP3/(dP5?) or dP2/(dP4/dP6?) common identity. Temporal morphogen signaling by BMPs may distinguish between these progenitor identities (Tozer et al., 2013), i.e. the dP2/dP4/dP6 state may arise from cells with limited exposure to BMP signaling, while the dP1/dP3/(dP5?) state arises from cells with more sustained exposure to BMP signaling. Discrete series of transcription factors are expressed in these domains and over time subdivide progenitor, and later neuronal, populations. For example, the basic-helix-loop-helix transcription factor, *Olig3*, under the regulation of BMP and Wnt signaling activity, is first expressed a broad dP1-dP3 domain (Zechner et al. 2007). Subsequently, *Atoh1* and *Ngn1* are expressed in the dP1 and dP2 domain respectively where they cross-repress one another. *Ngn1* also represses *Ascl1* which is expressed in the dP3-5 region (Gowan et al. 2001). The sequential activation/repression of these transcription factor states appear to be determined by early BMP patterning events, but then later affects the competency of the cells to respond to differentiation signals by the BMPs. Thus, following these early patterning events, cells in the dP1/dP3/(dP5?) state can respond to BMP4 to become either a dI1 or dI3, while the dP2/dP4/dP6 progenitor can only respond to BMP4 to become a dI2. Thus, prolonged addition of BMP4/RA to ESC cultures suppresses the dP2/dP4/dP6 state, such that no dI2s arise *in vitro*. In contrast, RA alone condition induces the dP2/dP4/dP6 state, permitting dI2s and small number of dI4/dI6s to be generated.

While these studies have shown that the BMPs are reiteratively used to pattern distinct progenitor and neuronal domains in the developing dorsal spinal cord, many mysteries still remain; BMP4 is not expressed in the mouse or human spinal cord, thus another BMP must be directing dorsal spinal identity in these species. Markers to distinguish between the dP1/dP3/dP5 versus dP2/dP4/dP6 state have not been identified. It is also unresolved how different types of dorsal INs arise from a common progenitor fate. BMP4 acts as a patterning and differentiation signal, it can specify dP1/dP3 progenitors and then reiteratively direct them to form either dI1s or dI3s. What are signals allow dP1/dP3 cells to determine whether to respond to BMP4 to become either a dI1 or dI3?

Finally, how are the dI4-dI6s, which are responsible for many important sensory modalities, specified? These cells appear to be dependent on RA signaling, as well as the transcription factor *Lbx1*, which is expressed in these populations. *Lbx1* is both necessary and sufficient to prevent dI4-dI6s from becoming dI2-dI4s (Gross et al., 2002; Muller et al., 2002). Thus, like the I-Smads, *Lbx1* may prevent the intermediate populations from interpreting the dorsal BMP signaling and thereby becoming more dorsalized.

### **Wnt signaling controls dorsal progenitor proliferation and patterning**

Like the BMPs, the Wnt family of signaling molecules is also large; 19 *Wnt* genes have been identified in the human genome, and are reiteratively used throughout development (Clevers and Nusse, 2012). Wnts regulate many cellular processes, aided by their ability to interact with other signaling pathways, including the mTOR, AKT CREB and PKC pathways, the core pathways that control cell proliferation, migration and survival as well as transcription (Logan and Nusse, 2004). Wnts signal through three known pathways: the canonical signaling pathway, where Wnt binding leads to the stabilization of the  $\beta$ -catenin second messenger, and two non-canonical pathways, the planar cell polarity (PCP) pathway and Wnt/calcium pathway, which are both  $\beta$ -catenin-independent. In the canonical Wnt pathway, the binding of Wnts to the frizzled (Fz) and LRP5/6 co-receptors, recruits disheveled (Dvl), and subsequently axin, to the signaling complex at the membrane (Cong et al., 2004; Hart et al., 1998). In the absence of Wnt signaling, axin is bound to a complex of proteins that degrades  $\beta$ -catenin. However, once axin is recruited to the membrane,  $\beta$ -catenin is stabilized and can translocate to the nucleus to act as transcriptional activator for Wnt target genes (Behrens et al., 1996; Tolwinski and Wieschaus, 2004). In contrast, the PCP pathway mediates cytoskeleton modification by activating actin regulators such as profilin and the Rho-associated protein kinase (ROCK) (Shulman et al., 1998) while the calcium pathway modulates G-protein regulated calcium release from the endoplasmic reticulum (Slusarski et al., 1997).

While Wnt ligands are critical for inductive patterning in the brain (McMahon and Bradley, 1990) and have a key role in the specification of the anterior-posterior axis (Agalliu et al., 2009; Green et al., 2015), relatively little is known about their role in patterning the dorsal-ventral axis of the spinal cord. At least four Wnts are expressed in the RP and dorsal spinal cord: Wnt1, Wnt3, Wnt3a and Wnt4 (Hollyday et al., 1995; Megason and McMahon, 2002; Parr et al., 1993) (Fig.

2A). Of these Wnts, Wnt1 and Wnt3a can act as mitogens to regulate cell proliferation during development of chicken spinal cord. Misexpression of either gene results in a ~2-fold increase in the number of cells in S-phase in the cell cycle (Megason and McMahon, 2002) and a general increase in the expression of dorsal markers (Alvarez-Medina et al., 2008). The loss-of-function experiments were performed in mice (Muroyama et al., 2002) and were consistent with the role of Wnt1 and Wnt3a as mitogens. They are genetically redundant; a single mutant in either Wnt had no effect on dorsal spinal identity. However, both dP1 and dP2 were depleted in *Wnt1; Wnt3a* double mutants, and there was profound reduction in the numbers of dI1s and dI3s. The concomitant increase in the dI4s suggests that Wnts might also have patterning activities, however this hypothesis requires further study. The mechanism that transduces these activities also remains unresolved. Wnt mediates its role as a mitogen through the canonical  $\beta$ -catenin pathway (Alvarez-Medina et al., 2008; Megason and McMahon, 2002). Inhibiting the Wnt transcriptional response resulting in an expansion of ventral gene expression at the expense of dorsal identities. This response was shown to be Gli-dependent, suggesting that Wnt signaling is also required to restrict the activity of Shh to the ventral spinal cord (Alvarez-Medina et al., 2008).

Taken together, these studies most strongly support the hypothesis that Wnts act as proliferative signals. Wnts are required for the appropriate proliferation of dorsal progenitors; without this mitogenic activity, fewer differentiated dorsal IN neurons are produced. It remains unresolved whether the Wnts directly specify neuronal identity in the dorsal spinal cord.

### **III. Looking ahead: what does knowledge of these manipulations mean for spinal cord regeneration?**

Spinal cord injuries (SCI) are a major public health concern. The spinal cord is particularly vulnerable to damage from either injury or disease because it has a relatively exposed position in the body and shares no functional redundancy with other structures in the CNS. Damage to the spinal cord can affect both sensory and motor systems below the injury site and can be debilitating emotionally and physically for patients. SCI patients often have reduced quality of life. The loss of motor circuits results in patients being unable to move. When the ability to process and relay sensory input to the brain is affected, patients can lose the capacity to sense and respond to external stimuli, like pain or temperature. They are at risk for serious burn injuries, from being unable to

detect noxious heat, and suffer the emotional consequences of being unable to receive reassuring touch. SCIs are estimated to affect over 1 million people in the US, and cost ~\$40 billion annually in health care (Abraira et al., 2017; Armour et al., 2016). SCI patients have significantly lower life expectancy than uninjured individuals, regardless of injury severity; their average life expectancy has not improved since the 1980s.

There is currently no treatment that can reverse damage to the spinal cord. While important progress has been made on neuromodulatory mechanisms, that can improve the function of the nerves remaining after SCI (AuYong and Lu, 2014), there is currently no means of reversing congenital or acute damage to the spinal cord. Such a treatment will require the regeneration of substantial amounts of tissue to concomitantly reestablish sensory and motor spinal circuitry. A tractable path towards that objective is use pluripotent stem cells (PSCs) to generate the necessary component neurons, which can then be used in cellular replacement therapies to replace lost tissue (Kadoya et al., 2016; Pawar et al., 2015; Piltti et al., 2015). PSCs are also a powerful reagent for understanding disease mechanisms and serving as a platform for drug screening.

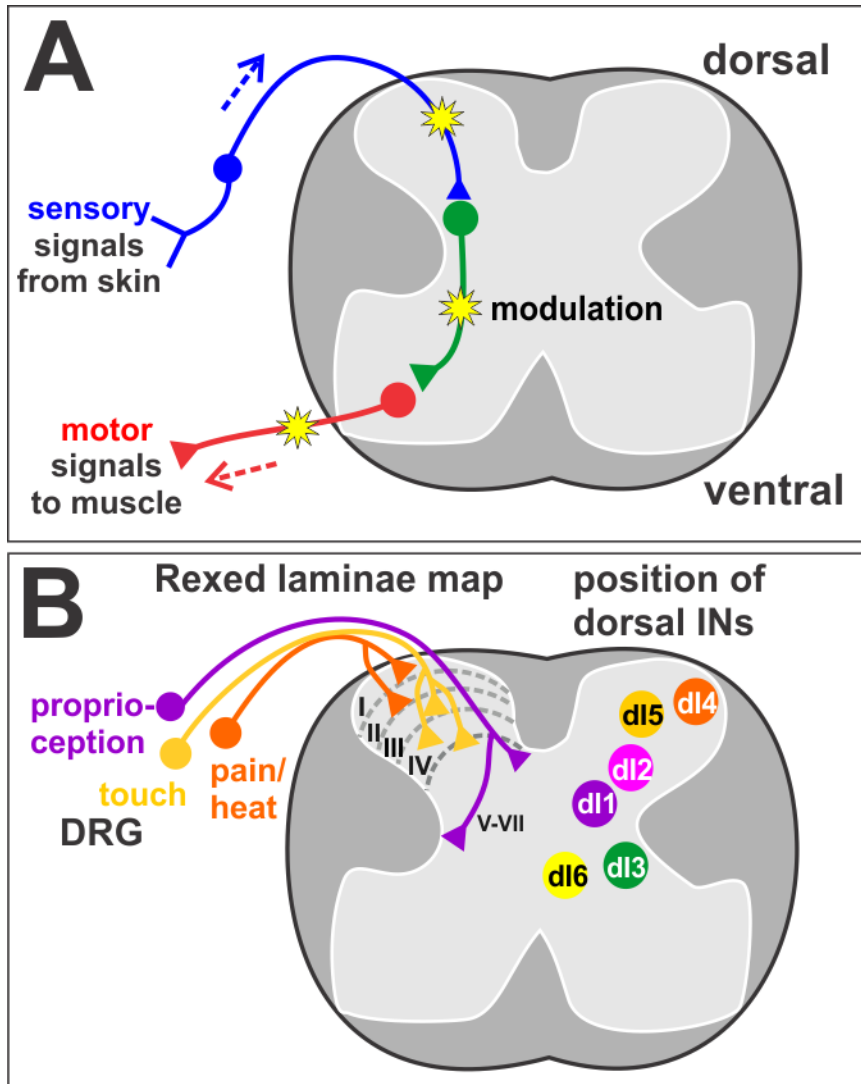
An important milestone towards a treatment for injured or diseased spinal cords has been the establishment of protocols to derive spinal MNs from both mouse and human ESCs (Li et al., 2005; Wichterle et al., 2002) and induced pluripotent stem cells (iPSCs) (Dimos et al., 2008; Karumbayaram et al., 2009). ESCs are directed toward a neurogenic lineage and caudalized by the addition of RA. Finally, they are ventralized by the addition of a Shh signaling agonist, such as purmorphamine, to specify spinal MN identity (Wichterle and Peljto, 2008). Stem cell-derived MNs have provided a critical window into understanding of the pathology of motor diseases, including amyotrophic lateral sclerosis (ALS) (Sances et al., 2016) and spinal muscular atrophy (SMA) (Ebert et al., 2009), as well permitting cellular replacement in injury models (Harper et al., 2004; Thomsen et al., 2014).

While these advances raise the possibility of repairing motor functions in SCI patients, this objective also requires the regeneration of the sensory circuits that modulate motor output. However, progress has been slowed by the lack of a directed differentiation protocol to generate sensory spinal relay INs from PSCs. We recently published the first directed differentiation

protocols for *in vitro* derived sensory spinal INs for both mESCs (Andrews et al., 2017) and hESCs and hiPSCs (Gupta et al., 2018). While BMP4 was used in the place of purmorphamine to dorsalize the identity of the RA-dependent spinal neurons, critical to the human protocol was the realization that has finding that the progenitors had an early window of competence to respond to BMP signaling to become dorsal neurons. These directed differentiation protocols can generate four key populations of dorsal sensory INs: the dI1s (proprioception), dI2s (unknown function), dI3s (touch-activated motor behaviors) and dI4/dI6s (pain/itch/touch) (Lai et al., 2016). These studies also established that hiPSCs can be directed to differentiate into dorsal sensory INs with comparable efficiency with hESCs.

Multiple challenges remain. While PSC-derived MNs have been shown to physiologically mirror their endogenous counterparts (Umbach et al., 2012), this outcome has yet to be demonstrated for PSC-derived dorsal sensory INs. Both PSC-derived MNs and dorsal INs arise as mixed populations, with varied efficiencies in the current protocols. It is critical to continue to work towards protocols that have the highest efficiency and purity of generating specific populations of spinal neurons. More studies also need to be performed to evaluate the ability to incorporate into the injured spinal cord of adult animal, form synaptic connections and provide functional recovery. However, there is now genuine hope that cellular replacement therapies can be successful used to regenerate complex tissues such as the spinal cord. Recent studies have shown that human-derived spinal neural precursors can successfully integrate into an injured non-human primate spinal cord resulting in some functional recovery (Rosenzweig et al., 2018). The ability to reverse congenital and acute damage to the spinal cord has the potential to impact millions of patients worldwide.

**Figure 1: Organization of the adult spinal cord**

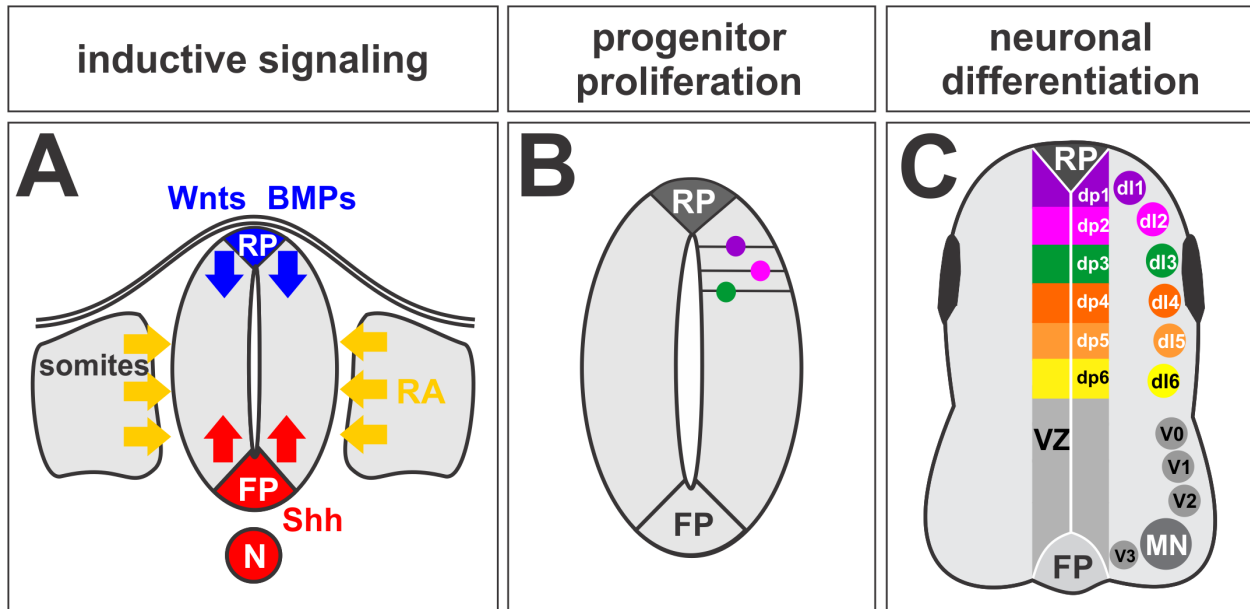


Schematics depicting the organization and function of the adult spinal cord.

(A) Sensory information from the peripheral nervous system, is relayed into the dorsal horn of the spinal cord, processed by interneurons in the dorsal and ventral spinal cord and then relayed to higher centers in the brains, or modulate motor function.

(B) The adult spinal cord is organized with a laminar architecture comprising seven distinct layers. Afferent sensory information is decoded in specific layers. Through fate mapping, genetic and connectivity studies, the general function and location of the dorsal IN populations have been identified in the adult spinal cord.

**Figure 2: Spinal cord development**



Schematics describing the stages of neuronal development in the spinal cord

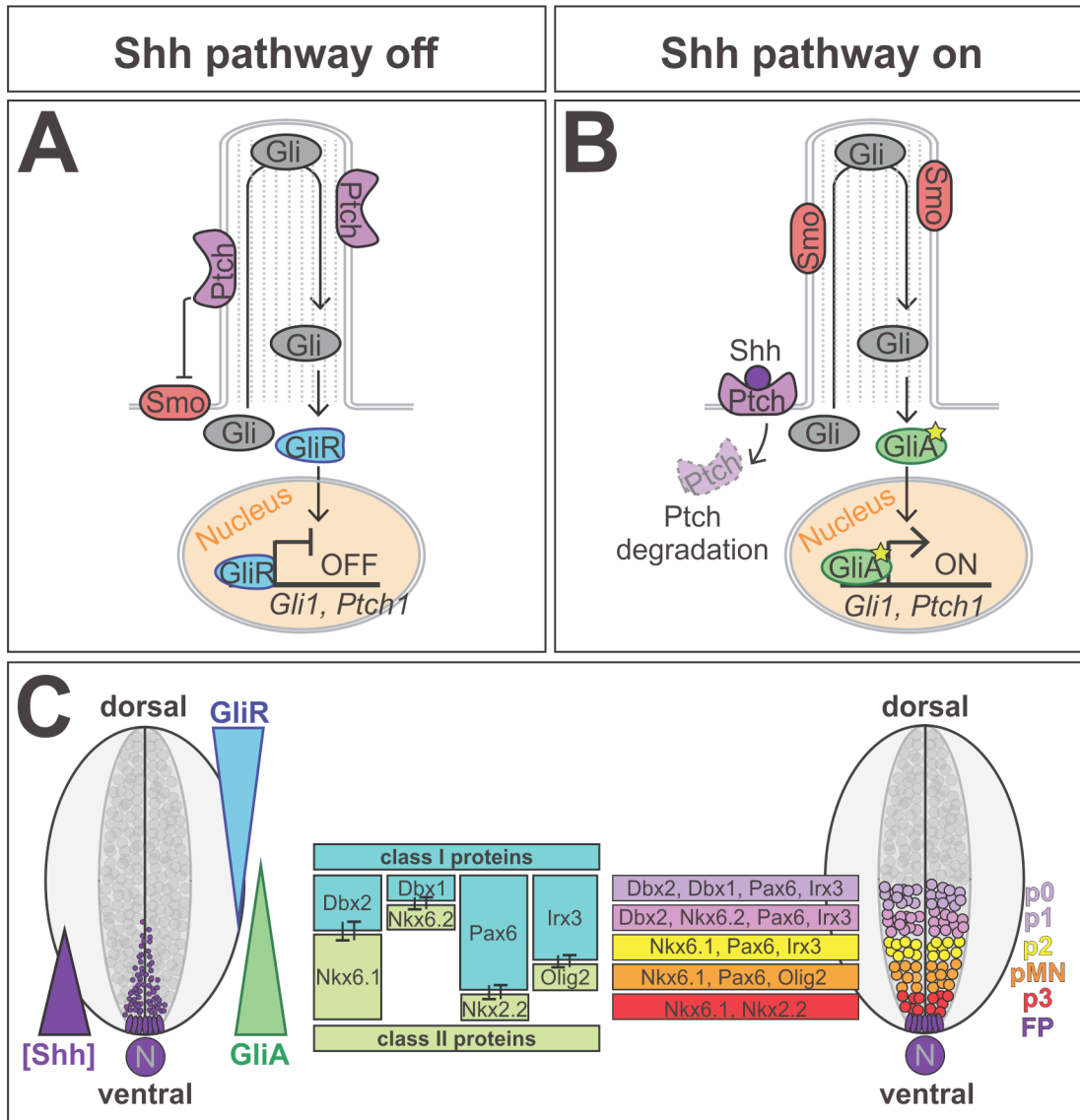
(A) Inductive signaling centers signal to spinal progenitors to pattern neural identity (modified with permission from (Tanabe and Jessell, 1996)). Sonic hedgehog (Shh) is secreted from the notochord and floor plate (FP) to pattern the ventral spinal cord. BMPs and Wnts are expressed from the roof plate (RP) to pattern the dorsal spinal cord. Retinoic acid (RA) is secreted from the somites in the paraxial mesoderm to specify axial level.

(B) During early spinal cord development, the nuclei of proliferating progenitors oscillate along the medial-lateral axis as they undergo cell division

(C) In response to inductive growth factors, progenitors are patterned into distinct domains within the ventricular zone (VZ) along the dorsal-ventral axis. As progenitors differentiate they exit the cell cycle and migrate to the lateral edge of the spinal cord. The dorsal spinal cord has six distinct interneurons (IN) populations, the dl1-6, while the ventral spinal cord has five distinct neuronal populations, the V0-V3 IN populations and the spinal MNs.



**Figure 3. Role of the Shh signaling pathway**

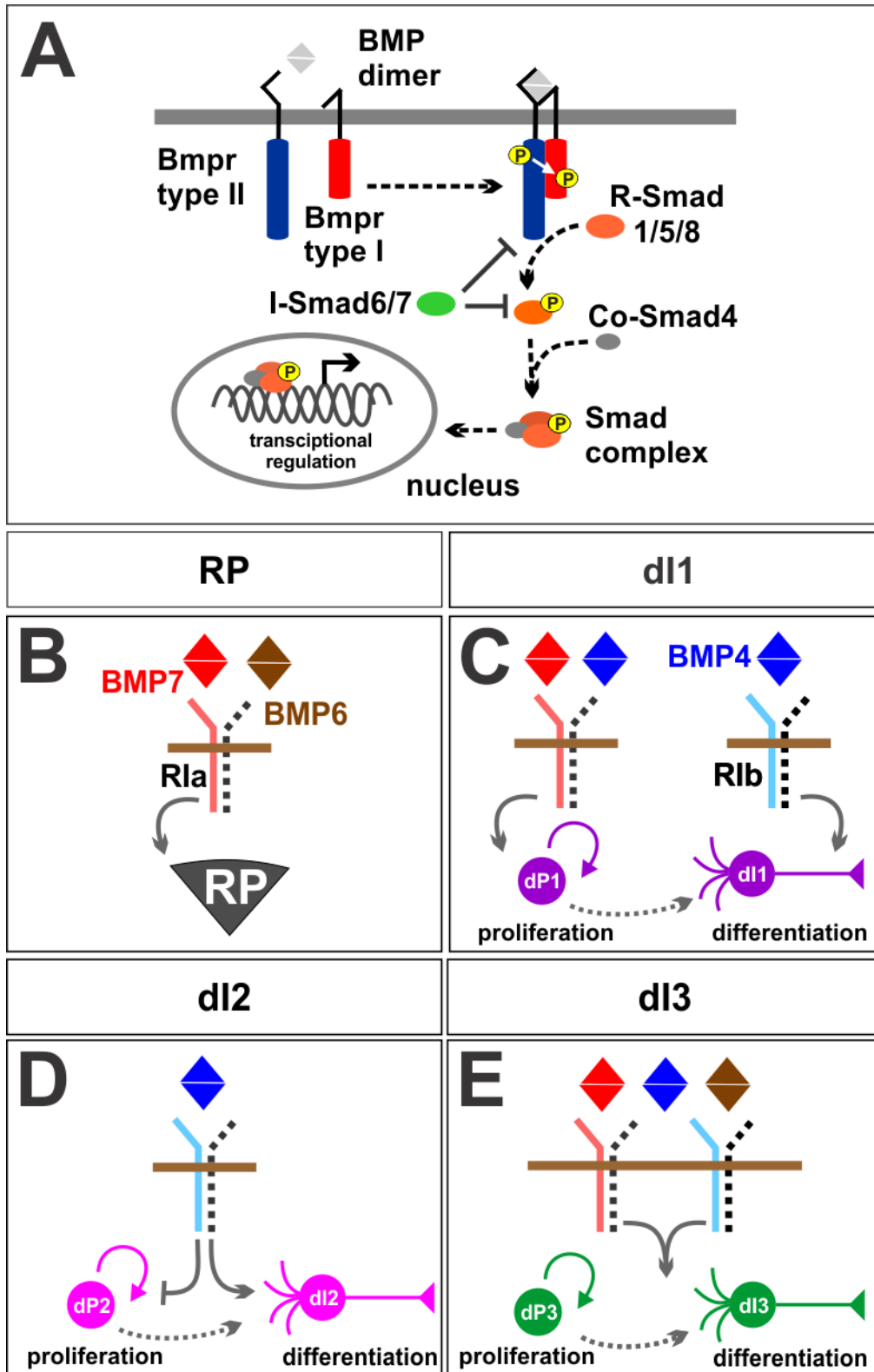


(A) Shh signal transduction (modified with permission from (Briscoe and Therond, 2013)). In the absence of Shh ligand, the patched (Ptch) accumulates at the base of, and within, the primary cilium. Through an indirect mechanism, the presence of Ptch represses the activation and movement of smoothed (Smo) into the primary cilium. In the absence of Smo, full length Gli proteins (Gli) are cleaved and processed into transcriptional repressors (GliR), which then translocate to the nucleus and repress downstream gene targets that include *Gli1* and *Ptch1*.

(B) In the presence of Shh, Shh ligand binds to Ptch, which is then is endocytosed and degraded. In the absence of Ptch, Smo is activated and translocates into the primary cilium. In the presence of Smo, Gli proteins are processed into transcriptional activators (GliA).

(C) Ventral spinal cord patterning (modified with permission from (Jessell, 2000; Oosterveen et al., 2012)). Shh ligand is secreted by the notochord (N) and the floor plate (FP) of the neural tube. Shh acts in a dose-dependent manner to alter the processing of Gli proteins from transcriptional repressors (GliR) to transcriptional activators (GliA). The net balance between GliA and GliR influences the expression of various homeodomain transcription factors along the dorsal-ventral axis. These transcription factors can be grouped into two classes: class I proteins (Dbx2, Dbx1, Pax6, and Irx3) that are influenced by RA signaling and present only in the absence of Shh and class II proteins (Nkx6.1, Nkx6.2, Nkx2.2, and Olig2) that require Shh for their activation. Ultimately, unique combinations of class I and class II transcription factors subdivide the ventral spinal cord into five molecularly distinct progenitor domains (p0, p1, p2, pMN, and p3). Over time, each of these progenitor domains give rise to distinct types of neurons and then glia.

Figure 4. BMP signaling pathway



(A) Schematic of BMP signaling (modified with permission from (Balemans and Van Hul, 2002)). BMP dimers bind to a complex of type I and type II receptors which phosphorylate receptor (R) activated Smads 1, 5 and 8. R-Smads complex with Co-Smad4 and enter the nucleus to regulate transcription. Inhibitory (I) Smads 6 and 7 negatively regulate the signaling pathway by competitively binding with other components of the signaling pathway.

(B-E) Model for a “mix and match” code for the specification of the RP, dI1s, dI2s and dI3s. In this model.

(B) BMP6 (mouse) and BMP7 (chicken) are the most effective at directing RP identity through the Bmpr1a receptor (mouse).

(C) Both BMP4 and BMP7 can promote dP1 patterning through Bmpr1a or Bmpr1b (chicken), but only BMP4 directs progenitors to differentiate as dI1s through Bmpr1b (mouse and chicken)).

(D) BMP4 also specifically directs dI2 differentiation in chicken, thereby depleting the pool of dP2s.

(E) All BMPs tested in both species, including BMP4, BMP5, BMP6 and BMP7, can act through either Bmpr1a or Bmpr1b to promote the dI3 fate.

## References

- Abraira, V.E., Kuehn, E.D., Chirila, A.M., Springel, M.W., Toliver, A.A., Zimmerman, A.L., Orefice, L.L., Boyle, K.A., Bai, L., Song, B.J., Bashista, K.A., O'Neill, T.G., Zhuo, J., Tsan, C., Hoynoski, J., Rutlin, M., Kus, L., Niederkofler, V., Watanabe, M., Dymecki, S.M., Nelson, S.B., Heintz, N., Hughes, D.I., Ginty, D.D., 2017. The Cellular and Synaptic Architecture of the Mechanosensory Dorsal Horn. *Cell* 168, 295-310 e219.
- Agalliu, D., Takada, S., Agalliu, I., McMahon, A.P., Jessell, T.M., 2009. Motor neurons with axial muscle projections specified by Wnt4/5 signaling. *Neuron* 61, 708-720.
- Ahn, K., Mishina, Y., Hanks, M.C., Behringer, R.R., Crenshaw, E.B., 3rd, 2001. BMPR-IA signaling is required for the formation of the apical ectodermal ridge and dorsal-ventral patterning of the limb. *Development* 128, 4449-4461.
- Alvarez-Medina, R., Cayuso, J., Okubo, T., Takada, S., Marti, E., 2008. Wnt canonical pathway restricts graded Shh/Gli patterning activity through the regulation of Gli3 expression. *Development* 135, 237-247.
- Andersson, L.S., Larhammar, M., Memic, F., Wootz, H., Schwochow, D., Rubin, C.J., Patra, K., Arnason, T., Wellbring, L., Hjalm, G., Imsland, F., Petersen, J.L., McCue, M.E., Mickelson, J.R., Cothran, G., Ahituv, N., Roepstorff, L., Mikko, S., Vallstedt, A., Lindgren, G., Andersson, L., Kullander, K., 2012. Mutations in DMRT3 affect locomotion in horses and spinal circuit function in mice. *Nature* 488, 642-646.
- Andrews, M.G., Del Castillo, L.M., Ochoa-Bolton, E., Yamauchi, K., Smogorzewski, J., Butler, S.J., 2017. BMPs direct sensory interneuron identity in the developing spinal cord using signal-specific not morphogenic activities. *eLife* 6.
- Arber, S., 2012. Motor circuits in action: specification, connectivity, and function. *Neuron* 74, 975-989.
- Armour, B.S., Courtney-Long, E.A., Fox, M.H., Fredine, H., Cahill, A., 2016. Prevalence and Causes of Paralysis-United States, 2013. *American journal of public health* 106, 1855-1857.
- AuYong, N., Lu, D.C., 2014. Neuromodulation of the lumbar spinal locomotor circuit. *Neurosurgery clinics of North America* 25, 15-23.
- Aza-Blanc, P., Lin, H.Y., Ruiz i Altaba, A., Kornberg, T.B., 2000. Expression of the vertebrate Gli proteins in *Drosophila* reveals a distribution of activator and repressor activities. *Development* 127, 4293-4301.
- Bai, C.B., Auerbach, W., Lee, J.S., Stephen, D., Joyner, A.L., 2002. Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. *Development* 129, 4753-4761.

- Balaskas, N., Ribeiro, A., Panovska, J., Dessaud, E., Sasai, N., Page, K.M., Briscoe, J., Ribes, V., 2012. Gene regulatory logic for reading the Sonic Hedgehog signaling gradient in the vertebrate neural tube. *Cell* 148, 273-284.
- Balemans, W., Van Hul, W., 2002. Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. *Dev Biol* 250, 231-250.
- Bautista, D.M., Siemens, J., Glazer, J.M., Tsuruda, P.R., Basbaum, A.I., Stucky, C.L., Jordt, S.E., Julius, D., 2007. The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature* 448, 204-208.
- Behrens, J., von Kries, J.P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., Birchmeier, W., 1996. Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* 382, 638-642.
- Bermingham, N.A., Hassan, B.A., Wang, V.Y., Fernandez, M., Banfi, S., Bellen, H.J., Fritsch, B., Zoghbi, H.Y., 2001. Proprioceptor pathway development is dependent on Math1. *Neuron* 30, 411-422.
- Bier, E., De Robertis, E.M., 2015. EMBRYO DEVELOPMENT. BMP gradients: A paradigm for morphogen-mediated developmental patterning. *Science* 348, aaa5838.
- Bourane, S., Duan, B., Koch, S.C., Dalet, A., Britz, O., Garcia-Campmany, L., Kim, E., Cheng, L., Ghosh, A., Ma, Q., Goulding, M., 2015a. Gate control of mechanical itch by a subpopulation of spinal cord interneurons. *Science* 350, 550-554.
- Bourane, S., Grossmann, K.S., Britz, O., Dalet, A., Del Barrio, M.G., Stam, F.J., Garcia-Campmany, L., Koch, S., Goulding, M., 2015b. Identification of a spinal circuit for light touch and fine motor control. *Cell* 160, 503-515.
- Briscoe, J., Novitsch, B.G., 2008. Regulatory pathways linking progenitor patterning, cell fates and neurogenesis in the ventral neural tube. *Philos Trans R Soc Lond B Biol Sci* 363, 57-70.
- Briscoe, J., Pierani, A., Jessell, T.M., 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., Ericson, J., 1999. Homeobox gene *Nkx2.2* and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* 398, 622-627.
- Briscoe, J., Therond, P.P., 2013. The mechanisms of Hedgehog signalling and its roles in development and disease. *Nat Rev Mol Cell Biol* 14, 416-429.
- Brown, A.G., Fyffe, R.E.W., 1981. Form and Function of Dorsal Horn Neurons with Axons Ascending the Dorsal Columns in Cat. *J Physiol-London* 321, 31-47.

- Bui, T.V., Akay, T., Loubani, O., Hnasko, T.S., Jessell, T.M., Brownstone, R.M., 2013. Circuits for grasping: spinal dI3 interneurons mediate cutaneous control of motor behavior. *Neuron* 78, 191-204.
- Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K.A., Dickson, B.J., Basler, K., 1999. Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. *Cell* 99, 803-815.
- Butler, S.J., Dodd, J., 2003. A role for BMP heterodimers in roof plate-mediated repulsion of commissural axons. *Neuron* 38, 389-401.
- Carpenter, D., Stone, D.M., Brush, J., Ryan, A., Armanini, M., Frantz, G., Rosenthal, A., de Sauvage, F.J., 1998. Characterization of two patched receptors for the vertebrate hedgehog protein family. *Proc Natl Acad Sci U S A* 95, 13630-13634.
- Casso, D.J., Biehs, B., Kornberg, T.B., 2011. A novel interaction between hedgehog and Notch promotes proliferation at the anterior-posterior organizer of the *Drosophila* wing. *Genetics* 187, 485-499.
- Catela, C., Shin, M.M., Dasen, J.S., 2015. Assembly and function of spinal circuits for motor control. *Annual review of cell and developmental biology* 31, 669-698.
- Chamberlain, C.E., Jeong, J., Guo, C., Allen, B.L., 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, Y., Struhl, G., 1996. Dual roles for patched in sequestering and transducing Hedgehog. *Cell* 87, 553-563.
- Chiang, C., Litington, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., Beachy, P.A., 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 383, 407-413.
- Chizhikov, V.V., Millen, K.J., 2004. Control of roof plate formation by *Lmx1a* in the developing spinal cord. *Development* 131, 2693-2705.
- Clevers, H., Nusse, R., 2012. Wnt/beta-catenin signaling and disease. *Cell* 149, 1192-1205.
- Cong, F., Schweizer, L., Varmus, H., 2004. Wnt signals across the plasma membrane to activate the beta-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP. *Development* 131, 5103-5115.
- Conlon, R.A., Rossant, J., 1992. Exogenous retinoic acid rapidly induces anterior ectopic expression of murine *Hox-2* genes in vivo. *Development* 116, 357-368.
- Corbit, K.C., Aanstad, P., Singla, V., Norman, A.R., Stainier, D.Y., Reiter, J.F., 2005. Vertebrate Smoothed functions at the primary cilium. *Nature* 437, 1018-1021.

- Crone, S.A., Quinlan, K.A., Zagoraïou, L., Droho, S., Restrepo, C.E., Lundfald, L., Endo, T., Setlak, J., Jessell, T.M., Kiehn, O., Sharma, K., 2008. Genetic ablation of V2a ipsilateral interneurons disrupts left-right locomotor coordination in mammalian spinal cord. *Neuron* 60, 70-83.
- Dasen, J.S., De Camilli, A., Wang, B., Tucker, P.W., Jessell, T.M., 2008. Hox repertoires for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. *Cell* 134, 304-316.
- Delacroix, L., Moutier, E., Altobelli, G., Legras, S., Poch, O., Choukrallah, M.A., Bertin, I., Jost, B., Davidson, I., 2010. Cell-specific interaction of retinoic acid receptors with target genes in mouse embryonic fibroblasts and embryonic stem cells. *Molecular and cellular biology* 30, 231-244.
- Dessaud, E., Ribes, V., Balaskas, N., Yang, L.L., Pierani, A., Kicheva, A., Novitch, B.G., Briscoe, J., Sasai, N., 2010. Dynamic assignment and maintenance of positional identity in the ventral neural tube by the morphogen sonic hedgehog. *PLoS Biol* 8, e1000382.
- Dessaud, E., Yang, L.L., Hill, K., Cox, B., Ulloa, F., Ribeiro, A., Mynett, A., Novitch, B.G., Briscoe, J., 2007. Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism. *Nature* 450, 717-720.
- Dickman, E.D., Thaller, C., Smith, S.M., 1997. Temporally-regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects. *Development* 124, 3111-3121.
- Dimos, J.T., Rodolfa, K.T., Niakan, K.K., Weisenthal, L.M., Mitumoto, H., Chung, W., Croft, G.F., Saphier, G., Leibel, R., Goland, R., Wichterle, H., Henderson, C.E., Eggan, K., 2008. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* 321, 1218-1221.
- Ducy, P., Karsenty, G., 2000. The family of bone morphogenetic proteins. *Kidney Int* 57, 2207-2214.
- Dudley, A.T., Lyons, K.M., Robertson, E.J., 1995. A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev* 9, 2795-2807.
- Durston, A.J., Timmermans, J.P., Hage, W.J., Hendriks, H.F., de Vries, N.J., Heideveld, M., Nieuwkoop, P.D., 1989. Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* 340, 140-144.
- Ebert, A.D., Yu, J., Rose, F.F., Jr., Mattis, V.B., Lorson, C.L., Thomson, J.A., Svendsen, C.N., 2009. Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature* 457, 277-280.
- Echelard, Y., Epstein, D.J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J.A., 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., 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., Briscoe, J., 1997b. Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* 90, 169-180.
- Gerhart, J., 1996. Johannes Holtfreter's contributions to ongoing studies of the organizer. *Developmental dynamics : an official publication of the American Association of Anatomists* 205, 245-256.
- Goetz, C., Pivetta, C., Arber, S., 2015. Distinct limb and trunk premotor circuits establish laterality in the spinal cord. *Neuron* 85, 131-144.
- Goetz, J.A., Singh, S., Suber, L.M., Kull, F.J., Robbins, D.J., 2006. A highly conserved amino-terminal region of sonic hedgehog is required for the formation of its freely diffusible multimeric form. *J Biol Chem* 281, 4087-4093.
- Goodrich, L.V., Johnson, R.L., Milenkovic, L., McMahon, J.A., Scott, M.P., 1996. Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev* 10, 301-312.
- Gosgnach, S., Bikoff, J.B., Dougherty, K.J., El Manira, A., Lanuza, G.M., Zhang, Y., 2017. Delineating the Diversity of Spinal Interneurons in Locomotor Circuits. *J Neurosci* 37, 10835-10841.
- Gosgnach, S., Lanuza, G.M., Butt, S.J., Saueressig, H., Zhang, Y., Velasquez, T., Riethmacher, D., Callaway, E.M., Kiehn, O., Goulding, M., 2006. V1 spinal neurons regulate the speed of vertebrate locomotor outputs. *Nature* 440, 215-219.
- Goulding, M., 2009. Circuits controlling vertebrate locomotion: moving in a new direction. *Nat Rev Neurosci* 10, 507-518.
- Green, D., Whitener, A.E., Mohanty, S., Lekven, A.C., 2015. Vertebrate nervous system posteriorization: Grading the function of Wnt signaling. *Developmental dynamics : an official publication of the American Association of Anatomists* 244, 507-512.
- Griener, A., Zhang, W., Kao, H., Wagner, C., Gosgnach, S., 2015. Probing diversity within subpopulations of locomotor-related V0 interneurons. *Developmental neurobiology* 75, 1189-1203.
- Gross, M.K., Dottori, M., Goulding, M., 2002. Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. *Neuron* 34, 535-549.
- Gupta, S., Sivalingam, D., Hain, S., Makkar, C., Sosa, E., Clark, A., Butler, S.J., 2018. Deriving Dorsal Spinal Sensory Interneurons from Human Pluripotent Stem Cells. *Stem cell reports* 10, 390-405.

- Hallahan, A.R., Pritchard, J.I., Hansen, S., Benson, M., Stoeck, J., Hatton, B.A., Russell, T.L., Ellenbogen, R.G., Bernstein, I.D., Beachy, P.A., Olson, J.M., 2004. The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas. *Cancer research* 64, 7794-7800.
- Hamidi, H., Gustafson, D., Pellegrini, M., Gasson, J., 2011. Identification of novel targets of CSL-dependent Notch signaling in hematopoiesis. *PloS one* 6, e20022.
- Hammerschmidt, M., Brook, A., McMahon, A.P., 1997. The world according to hedgehog. *Trends Genet* 13, 14-21.
- Harper, J.M., Krishnan, C., Darman, J.S., Deshpande, D.M., Peck, S., Shats, I., Backovic, S., Rothstein, J.D., Kerr, D.A., 2004. Axonal growth of embryonic stem cell-derived motoneurons in vitro and in motoneuron-injured adult rats. *Proc Natl Acad Sci U S A* 101, 7123-7128.
- Hart, M.J., de los Santos, R., Albert, I.N., Rubinfeld, B., Polakis, P., 1998. Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. *Curr Biol* 8, 573-581.
- Hazen, V.M., Andrews, M.A., Umans, L., Crenshaw, E.B., 3rd, Zwijsen, A., Butler, S.J., 2012. BMP receptor-activated Smads direct diverse functions during the development of the dorsal spinal cord. *Dev Biol* 367, 216-227.
- Hazen, V.M., Phan, K.D., Hudiburgh, S., Butler, S.J., 2011. Inhibitory Smads differentially regulate cell fate specification and axon dynamics in the dorsal spinal cord. *Dev Biol* 356, 566-575.
- Helms, A.W., Johnson, J.E., 2003. Specification of dorsal spinal cord interneurons. *Curr Opin Neurobiol* 13, 42-49.
- Hogan, B.L., 1996. Bone morphogenetic proteins in development. *Curr Opin Genet Dev* 6, 432-438.
- Hollyday, M., McMahon, J.A., McMahon, A.P., 1995. Wnt expression patterns in chick embryo nervous system. *Mech Dev* 52, 9-25.
- Huang, D., Chen, S.W., Gudas, L.J., 2002. Analysis of two distinct retinoic acid response elements in the homeobox gene Hoxb1 in transgenic mice. *Developmental dynamics : an official publication of the American Association of Anatomists* 223, 353-370.
- Huang, P., Xiong, F., Megason, S.G., Schier, A.F., 2012. Attenuation of Notch and Hedgehog signaling is required for fate specification in the spinal cord. *PLoS genetics* 8, e1002762.
- Hui, C.C., Angers, S., 2011. Gli proteins in development and disease. *Annual review of cell and developmental biology* 27, 513-537.
- Hui, C.C., Slusarski, D., Platt, K.A., Holmgren, R., Joyner, A.L., 1994. Expression of three mouse homologs of the *Drosophila* segment polarity gene cubitus interruptus, Gli, Gli-2, and

Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev Biol* 162, 402-413.

Hynes, M., Stone, D.M., Dowd, M., Pitts-Meek, S., Goddard, A., Gurney, A., Rosenthal, A., 1997. Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli-1. *Neuron* 19, 15-26.

Ingham, P.W., Taylor, A.M., Nakano, Y., 1991. Role of the *Drosophila* patched gene in positional signalling. *Nature* 353, 184-187.

Jeong, J., McMahon, A.P., 2005. Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1. *Development* 132, 143-154.

Jessell, T.M., 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat Rev Genet* 1, 20-29.

Jessell, T.M., Surmeli, G., Kelly, J.S., 2011. Motor neurons and the sense of place. *Neuron* 72, 419-424.

Kadoya, K., Lu, P., Nguyen, K., Lee-Kubli, C., Kumamaru, H., Yao, L., Knackert, J., Poplawski, G., Dulin, J.N., Strobl, H., Takashima, Y., Biane, J., Conner, J., Zhang, S.C., Tuszynski, M.H., 2016. Spinal cord reconstitution with homologous neural grafts enables robust corticospinal regeneration. *Nat Med* 22, 479-487.

Kam, R.K., Deng, Y., Chen, Y., Zhao, H., 2012. Retinoic acid synthesis and functions in early embryonic development. *Cell & bioscience* 2, 11.

Karumbayaram, S., Novitch, B.G., Patterson, M., Umbach, J.A., Richter, L., Lindgren, A., Conway, A.E., Clark, A.T., Goldman, S.A., Plath, K., Wiedau-Pazos, M., Kornblum, H.I., Lowry, W.E., 2009. Directed differentiation of human-induced pluripotent stem cells generates active motor neurons. *Stem cells* 27, 806-811.

Kong, J.H., Yang, L., Dessaud, E., Chuang, K., Moore, D.M., Rohatgi, R., Briscoe, J., Novitch, B.G., 2015. Notch activity modulates the responsiveness of neural progenitors to sonic hedgehog signaling. *Dev Cell* 33, 373-387.

Koop, D., Holland, N.D., Semon, M., Alvarez, S., de Lera, A.R., Laudet, V., Holland, L.Z., Schubert, M., 2010. Retinoic acid signaling targets Hox genes during the amphioxus gastrula stage: insights into early anterior-posterior patterning of the chordate body plan. *Dev Biol* 338, 98-106.

Krauss, S., Concordet, J.P., Ingham, P.W., 1993. A functionally conserved homolog of the *Drosophila* segment polarity gene *hh* is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* 75, 1431-1444.

- Kutejova, E., Sasai, N., Shah, A., Gouti, M., Briscoe, J., 2016. Neural Progenitors Adopt Specific Identities by Directly Repressing All Alternative Progenitor Transcriptional Programs. *Dev Cell* 36, 639-653.
- Lai, H.C., Seal, R.P., Johnson, J.E., 2016. Making sense out of spinal cord somatosensory development. *Development* 143, 3434-3448.
- Le Dreau, G., Garcia-Campmany, L., Rabadan, M.A., Ferronha, T., Tozer, S., Briscoe, J., Marti, E., 2012. Canonical BMP7 activity is required for the generation of discrete neuronal populations in the dorsal spinal cord. *Development* 139, 259-268.
- Le Dreau, G., Marti, E., 2013. The multiple activities of BMPs during spinal cord development. *Cellular and molecular life sciences : CMLS* 70, 4293-4305.
- Lee, J., Platt, K.A., Censullo, P., Ruiz i Altaba, A., 1997. Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. *Development* 124, 2537-2552.
- Lee, J.J., von Kessler, D.P., Parks, S., Beachy, P.A., 1992. Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene hedgehog. *Cell* 71, 33-50.
- Lee, K.J., Jessell, T.M., 1999. The specification of dorsal cell fates in the vertebrate central nervous system. *Annu Rev Neurosci* 22, 261-294.
- Lee, K.J., Mendelsohn, M., Jessell, T.M., 1998. Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. *Genes Dev* 12, 3394-3407.
- Li, X.J., Du, Z.W., Zarnowska, E.D., Pankratz, M., Hansen, L.O., Pearce, R.A., Zhang, S.C., 2005. Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol* 23, 215-221.
- Li, Y., Hibbs, M.A., Gard, A.L., Shylo, N.A., Yun, K., 2012. Genome-wide analysis of N1ICD/RBPJ targets in vivo reveals direct transcriptional regulation of Wnt, SHH, and hippo pathway effectors by Notch1. *Stem cells* 30, 741-752.
- Liem, K.F., Jr., Tremml, G., Jessell, T.M., 1997. A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. *Cell* 91, 127-138.
- Liem, K.F., Tremml, G., Roelink, H., Jessell, T.M., 1995. Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* 82, 969-979.
- Logan, C.Y., Nusse, R., 2004. The Wnt signaling pathway in development and disease. *Annual review of cell and developmental biology* 20, 781-810.
- Lopez, S.L., Paganelli, A.R., Siri, M.V., Ocana, O.H., Franco, P.G., Carrasco, A.E., 2003. Notch activates sonic hedgehog and both are involved in the specification of dorsal midline cell-fates in *Xenopus*. *Development* 130, 2225-2238.

- Lyons, K.M., Hogan, B.L., Robertson, E.J., 1995. Colocalization of BMP 7 and BMP 2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development. *Mech Dev* 50, 71-83.
- Maden, M., 2002. Retinoid signalling in the development of the central nervous system. *Nat Rev Neurosci* 3, 843-853.
- Maden, M., 2007. Retinoic acid in the development, regeneration and maintenance of the nervous system. *Nat Rev Neurosci* 8, 755-765.
- Marigo, V., Davey, R.A., Zuo, Y., Cunningham, J.M., Tabin, C.J., 1996. Biochemical evidence that patched is the Hedgehog receptor. *Nature* 384, 176-179.
- Massague, J., Seoane, J., Wotton, D., 2005. Smad transcription factors. *Genes Dev* 19, 2783-2810.
- McMahon, A.P., Bradley, A., 1990. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* 62, 1073-1085.
- Megason, S.G., McMahon, A.P., 2002. A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* 129, 2087-2098.
- Miesegeaes, G.R., Klisch, T.J., Thaller, C., Ahmad, K.A., Atkinson, R.C., Zoghbi, H.Y., 2009. Identification and subclassification of new Atoh1 derived cell populations during mouse spinal cord development. *Dev Biol* 327, 339-351.
- Mohler, J., Vani, K., 1992. Molecular organization and embryonic expression of the hedgehog gene involved in cell-cell communication in segmental patterning of *Drosophila*. *Development* 115, 957-971.
- Moustakas, A., Heldin, C.H., 2009. The regulation of TGFbeta signal transduction. *Development* 136, 3699-3714.
- Muller, T., Brohmann, H., Pierani, A., Heppenstall, P.A., Lewin, G.R., Jessell, T.M., Birchmeier, C., 2002. The homeodomain factor *lhx1* distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. *Neuron* 34, 551-562.
- Muroyama, Y., Fujihara, M., Ikeya, M., Kondoh, H., Takada, S., 2002. Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord. *Genes Dev* 16, 548-553.
- Nguyen, V.H., Trout, J., Connors, S.A., Andermann, P., Weinberg, E., Mullins, M.C., 2000. Dorsal and intermediate neuronal cell types of the spinal cord are established by a BMP signaling pathway. *Development* 127, 1209-1220.

- Niewiadomski P, Kong JH, Ahrends R, Ma Y, Humke EW, Khan S, Teruel MN, Novitch BG, Rohatgi R. 2014. Gli protein activity is controlled by multisite phosphorylation in vertebrate Hedgehog signaling. *Cell Rep* 6, 168-181.
- Novitch, B.G., Wichterle, H., Jessell, T.M., Sockanathan, S., 2003. A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. *Neuron* 40, 81-95.
- Nusslein-Volhard, C., Wieschaus, E., 1980. Mutations affecting segment number and polarity in *Drosophila*. *Nature* 287, 795-801.
- Okada, Y., Shimazaki, T., Sobue, G., Okano, H., 2004. Retinoic-acid-concentration-dependent acquisition of neural cell identity during in vitro differentiation of mouse embryonic stem cells. *Dev Biol* 275, 124-142.
- Oosterveen, T., Kurdija, S., Alekseenko, Z., Uhde, C.W., Bergsland, M., Sandberg, M., Andersson, E., Dias, J.M., Muhr, J., Ericson, J., 2012. Mechanistic differences in the transcriptional interpretation of local and long-range Shh morphogen signaling. *Dev Cell* 23, 1006-1019.
- Pankratz, M.J., Jäckle, H., 1990. Making stripes in the *Drosophila* embryo. *Trends Genet* 6, 287-292.
- Parr, B.A., Shea, M.J., Vassileva, G., McMahon, A.P., 1993. Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* 119, 247-261.
- Pawar, K., Cummings, B.J., Thomas, A., Shea, L.D., Levine, A., Pfaff, S., Anderson, A.J., 2015. Biomaterial bridges enable regeneration and re-entry of corticospinal tract axons into the caudal spinal cord after SCI: Association with recovery of forelimb function. *Biomaterials* 65, 1-12.
- Persson, M., Stamataki, D., te Welscher, P., Andersson, E., Bose, J., Ruther, U., Ericson, J., Briscoe, J., 2002. Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity. *Genes Dev* 16, 2865-2878.
- Piltti, K.M., Avakian, S.N., Funes, G.M., Hu, A., Uchida, N., Anderson, A.J., Cummings, B.J., 2015. Transplantation dose alters the dynamics of human neural stem cell engraftment, proliferation and migration after spinal cord injury. *Stem Cell Res* 15, 341-353.
- Rexed, B., 1954. A cytoarchitectonic atlas of the spinal cord in the cat. *The Journal of comparative neurology* 100, 297-379.
- Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T.M., et al., 1994. Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* 76, 761-775.
- Roelink, H., Porter, J.A., Chiang, C., Tanabe, Y., Chang, D.T., Beachy, P.A., 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., Scott, M.P., 2007. Patched1 regulates hedgehog signaling at the primary cilium. *Science* 317, 372-376.
- Rosenzweig, E.S., Brock, J.H., Lu, P., Kumamaru, H., Salegio, E.A., Kadoya, K., Weber, J.L., Liang, J.J., Moseanko, R., Hawbecker, S., Huie, J.R., Havton, L.A., Nout-Lomas, Y.S., Ferguson, A.R., Beattie, M.S., Bresnahan, J.C., Tuszynski, M.H., 2018. Restorative effects of human neural stem cell grafts on the primate spinal cord. *Nat Med* 24, 484-490.
- Rousso, D.L., Gaber, Z.B., Wellik, D., Morrisey, E.E., Novitch, B.G., 2008. Coordinated actions of the forkhead protein Foxp1 and Hox proteins in the columnar organization of spinal motor neurons. *Neuron* 59, 226-240.
- Rowitch DH, Kriegstein AR. 2010. Developmental genetics of vertebrate glial-cell specification. *Nature* 468, 214-222.
- Ruiz i Altaba, A., 1998. Combinatorial Gli gene function in floor plate and neuronal inductions by Sonic hedgehog. *Development* 125, 2203-2212.
- Sagner, A., Gaber, Z.B., Delile, J., Kong, J.H., Rousso, D.L., Pearson, C.A., Weicksel, S.E., Melchionda, M., Mousavy Gharavy, S.N., Briscoe, J., Novitch, B.G., 2018. Olig2 and Hes regulatory dynamics during motor neuron differentiation revealed by single cell transcriptomics. *PLoS Biol* 16, e2003127.
- Sances, S., Bruijn, L.I., Chandran, S., Eggan, K., Ho, R., Klim, J.R., Livesey, M.R., Lowry, E., Macklis, J.D., Rushton, D., Sadegh, C., Sareen, D., Wichterle, H., Zhang, S.C., Svendsen, C.N., 2016. Modeling ALS with motor neurons derived from human induced pluripotent stem cells. *Nature neuroscience* 19, 542-553.
- Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M., Kondoh, H., 1999. Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* 126, 3915-3924.
- Sharpe, C.R., Goldstone, K., 1997. Retinoid receptors promote primary neurogenesis in *Xenopus*. *Development* 124, 515-523.
- Shi, Y., Massague, J., 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 113, 685-700.
- Shulman, J.M., Perrimon, N., Axelrod, J.D., 1998. Frizzled signaling and the developmental control of cell polarity. *Trends Genet* 14, 452-458.
- Slusarski, D.C., Yang-Snyder, J., Busa, W.B., Moon, R.T., 1997. Modulation of embryonic intracellular Ca<sup>2+</sup> signaling by Wnt-5A. *Dev Biol* 182, 114-120.
- Stamatakis, D., Ulloa, F., Tsoni, S.V., Mynett, A., Briscoe, J., 2005. A gradient of Gli activity mediates graded Sonic Hedgehog signaling in the neural tube. *Genes Dev* 19, 626-641.

- Stasiulewicz, M., Gray, S.D., Mastromina, I., Silva, J.C., Bjorklund, M., Seymour, P.A., Booth, D., Thompson, C., Green, R.J., Hall, E.A., Serup, P., Dale, J.K., 2015. A conserved role for Notch signaling in priming the cellular response to Shh through ciliary localisation of the key Shh transducer Smo. *Development* 142, 2291-2303.
- Stone, D.M., Hynes, M., Armanini, M., Swanson, T.A., Gu, Q., Johnson, R.L., Scott, M.P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J.E., de Sauvage, F., Rosenthal, A., 1996. The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* 384, 129-134.
- Szabo, N.E., da Silva, R.V., Sotocinal, S.G., Zeilhofer, H.U., Mogil, J.S., Kania, A., 2015. Hoxb8 intersection defines a role for Lmx1b in excitatory dorsal horn neuron development, spinofugal connectivity, and nociception. *J Neurosci* 35, 5233-5246.
- Tabata, T., Eaton, S., Kornberg, T.B., 1992. The *Drosophila* hedgehog gene is expressed specifically in posterior compartment cells and is a target of engrailed regulation. *Genes Dev* 6, 2635-2645.
- Tanabe, Y., Jessell, T.M., 1996. Diversity and pattern in the developing spinal cord. *Science* 274, 1115-1123.
- Tashiro, S., Michiue, T., Higashijima, S., Zenno, S., Ishimaru, S., Takahashi, F., Orihara, M., Kojima, T., Saigo, K., 1993. Structure and expression of hedgehog, a *Drosophila* segment-polarity gene required for cell-cell communication. *Gene* 124, 183-189.
- ten Dijke, P., Yamashita, H., Sampath, T.K., Reddi, A.H., Estevez, M., Riddle, D.L., Ichijo, H., Heldin, C.H., Miyazono, K., 1994. Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. *J Biol Chem* 269, 16985-16988.
- Thomsen, G.M., Gowing, G., Svendsen, S., Svendsen, C.N., 2014. The past, present and future of stem cell clinical trials for ALS. *Exp Neurol* 262 Pt B, 127-137.
- Timmer, J., Chesnutt, C., Niswander, L., 2005. The activin signaling pathway promotes differentiation of dI3 interneurons in the spinal neural tube. *Dev Biol* 285, 1-10.
- Timmer, J.R., Wang, C., 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.
- Todd, A.J., 2010. Neuronal circuitry for pain processing in the dorsal horn. *Nat Rev Neurosci* 11, 823-836.
- Tolwinski, N.S., Wieschaus, E., 2004. A nuclear function for armadillo/beta-catenin. *PLoS Biol* 2, E95.
- Tozer, S., Le Dreau, G., Marti, E., Briscoe, J., 2013. Temporal control of BMP signalling determines neuronal subtype identity in the dorsal neural tube. *Development* 140, 1467-1474.



- Umbach, J.A., Adams, K.L., Gundersen, C.B., Novitch, B.G., 2012. Functional neuromuscular junctions formed by embryonic stem cell-derived motor neurons. *PloS one* 7, e36049.
- Urist, M.R., Dowell, T.A., Hay, P.H., Strates, B.S., 1968. Inductive substrates for bone formation. *Clin Orthop Relat Res* 59, 59-96.
- Varjosalo, M., Taipale, J., 2008. Hedgehog: functions and mechanisms. *Genes Dev* 22, 2454-2472.
- Villeda, S.A., Akopians, A.L., Babayan, A.H., Basbaum, A.I., Phelps, P.E., 2006. Absence of Reelin results in altered nociception and aberrant neuronal positioning in the dorsal spinal cord. *Neuroscience* 139, 1385-1396.
- Wada, H., Makabe, K., 2006. Genome duplications of early vertebrates as a possible chronicle of the evolutionary history of the neural crest. *International journal of biological sciences* 2, 133-141.
- Wichterle, H., Lieberam, I., Porter, J.A., Jessell, T.M., 2002. Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110, 385-397.
- Wichterle, H., Peljto, M., 2008. Differentiation of mouse embryonic stem cells to spinal motor neurons. *Current protocols in stem cell biology* Chapter 1, Unit 1H 1 1-1H 1 9.
- Wilson, L., Gale, E., Chambers, D., Maden, M., 2004. Retinoic acid and the control of dorsoventral patterning in the avian spinal cord. *Dev Biol* 269, 433-446.
- Wine-Lee, L., Ahn, K.J., Richardson, R.D., Mishina, Y., Lyons, K.M., Crenshaw, E.B., 3rd, 2004. Signaling through BMP type 1 receptors is required for development of interneuron cell types in the dorsal spinal cord. *Development* 131, 5393-5403.
- Wolpert, L., 1969. Positional information and the spatial pattern of cellular differentiation. *J Theor Biol* 25, 1-47.
- Wu, M.Y., Hill, C.S., 2009. Tgf-beta superfamily signaling in embryonic development and homeostasis. *Dev Cell* 16, 329-343.
- Wyatt, A.W., Osborne, R.J., Stewart, H., Ragge, N.K., 2010. Bone morphogenetic protein 7 (BMP7) mutations are associated with variable ocular, brain, ear, palate, and skeletal anomalies. *Hum Mutat* 31, 781-787.
- Xu, Y., Lopes, C., Wende, H., Guo, Z., Cheng, L., Birchmeier, C., Ma, Q., 2013. Ontogeny of excitatory spinal neurons processing distinct somatic sensory modalities. *J Neurosci* 33, 14738-14748.
- Yamada, T., Pfaff, S.L., Edlund, T., Jessell, T.M., 1993. Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. *Cell* 73, 673-686.

- Yamada, T., Placzek, M., Tanaka, H., Dodd, J., Jessell, T.M., 1991. Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. *Cell* 64, 635-647.
- Yamauchi, K., Phan, K.D., Butler, S.J., 2008. BMP type I receptor complexes have distinct activities mediating cell fate and axon guidance decisions. *Development* 135, 1119-1128.
- Yang, Q., Bermingham, N.A., Finegold, M.J., Zoghbi, H.Y., 2001. Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. *Science* 294, 2155-2158.
- Yasaka, T., Tiong, S.Y., Hughes, D.I., Riddell, J.S., Todd, A.J., 2010. Populations of inhibitory and excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined electrophysiological and anatomical approach. *Pain* 151, 475-488.
- Yuengert, R., Hori, K., Kibodeaux, E.E., McClellan, J.X., Morales, J.E., Huang, T.W., Neul, J.L., Lai, H.C., 2015. Origin of a Non-Clarke's Column Division of the Dorsal Spinocerebellar Tract and the Role of Caudal Proprioceptive Neurons in Motor Function. *Cell reports* 13, 1258-1271.
- Zeng, X., Goetz, J.A., Suber, L.M., Scott, W.J., Jr., Schreiner, C.M., Robbins, D.J., 2001. A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature* 411, 716-720.
- Zhang, J., Lanuza, G.M., Britz, O., Wang, Z., Siembab, V.C., Zhang, Y., Velasquez, T., Alvarez, F.J., Frank, E., Goulding, M., 2014. V1 and v2b interneurons secure the alternating flexor-extensor motor activity mice require for limbed locomotion. *Neuron* 82, 138-150.
- Zhang, Y., Narayan, S., Geiman, E., Lanuza, G.M., Velasquez, T., Shanks, B., Akay, T., Dyck, J., Pearson, K., Gosgnach, S., Fan, C.M., Goulding, M., 2008. V3 spinal neurons establish a robust and balanced locomotor rhythm during walking. *Neuron* 60, 84-96.