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A homeodomain feedback circuit underlies step-function interpretation of a Shh morphogen gradient during ventral neural patterning

Madelen Lek[1,*,†], José M. Dias[1,†], Ulrika Marklund[1], Christopher W. Uhde[1], Sanja Kurdija[1], Qiubo Lei[2], Lori Sussel[3], John L. Rubenstein[4], Michael P. Matise[5], Hans-Henning Arnold[6], Thomas M. Jessell[7] and Johan Ericson[1,‡]

SUMMARY

The deployment of morphogen gradients is a core strategy to establish cell diversity in developing tissues, but little is known about how small differences in the concentration of extracellular signals are translated into robust patterning output in responding cells. We have examined the activity of homeodomain proteins, which are presumed to operate downstream of graded Shh signaling in neural patterning, and describe a feedback circuit between the Shh pathway and homeodomain transcription factors that establishes non-graded regulation of Shh signaling activity. Nkx2 proteins intrinsically strengthen Shh responses in a feed-forward amplification and are required for ventral floor plate and p3 progenitor fates. Conversely, Pax6 has an opposing function to antagonize Shh signaling, which provides intrinsic resistance to Shh responses and is important to constrain the inductive capacity of the Shh gradient over time. Our data further suggest that patterning of floor plate cells and p3 progenitors is gated by a temporal switch in neuronal potential, rather than by different Shh concentrations. These data establish that dynamic, non-graded changes in responding cells are essential for Shh morphogen interpretation, and provide a rationale to explain mechanistically the phenomenon of cellular memory of morphogen exposure.

KEY WORDS: Floor plate, Shh, Homeodomain, Nkx2.2, Pax6, Gli, Morphogen

INTRODUCTION

A general strategy to establish cellular diversity in developing organisms is the deployment of morphogen gradients that signal at long range from localized sources to impart positional information to surrounding cells (Lander, 2007). A well-studied example in vertebrate development is the secreted protein Sonic hedgehog (Shh), which is responsible for conveying such information to cells of the ventral neural tube (Jessell, 2000) and to other tissues such as the limb bud (Bastida and Ros, 2008). In the neural tube, this activity is manifested in the patterned generation of distinct neuronal subtypes at stereotypic positions in response to graded Shh signaling by ventral midline cells. A key role for Shh in this process is to regulate expression of patterning transcription factors, but the mechanisms by which information is translated from the extracellular Shh gradient into a precise and robust transcriptional output remain poorly understood.

Models of graded Shh signaling in the neural tube (Roelink et al., 1995; Ericson et al., 1997; Dessaud et al., 2007) posit that a long-range extrinsic spatiotemporal gradient of ligand emanates first from the notochord and then from the floor plate (FP). The Shh signal is received by neural progenitors and transduced by the Patched/Smoothened (Ptc1/Smo)-receptor complex, ultimately regulating activity of the executors of the Shh pathway, zinc-finger domain-containing transcription factors of the Gli family. In the absence of Shh, Gli2 and Gli3 are processed to transcriptional repressors (GliR), whereas both are stabilized in their activator forms (GliA) upon exposure to Shh ligand (reviewed by Dessaud et al., 2008). Gli proteins regulate the patterned expression of homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors in neural progenitors, and the cross-repressive interactions between these proteins are important for the establishment and maintenance of ventral progenitor domains expressing distinctive molecular profiles (Briscoe et al., 2000; Muhr et al., 2001). These transcription factors provide positional value to cells and hence determine the identity of neuronal subtypes generated from distinct progenitor populations (Dessaud et al., 2008).

Evidence for graded activity of Shh originates primarily from ex vivo experiments, in which incremental two- to threefold increases in the ambient Shh concentration (Roelink et al., 1995; Ericson et al., 1997) or in the duration of exposure to Shh (Dessaud et al., 2007) result in induction of cell types that are characteristic of progressively more ventral regions of the neural tube. Shh signaling

[1]Department of Cell and Molecular Biology, Karolinska Institutet, S-17177 Stockholm, Sweden. [2]Sanofi-Aventis US, 1041 Route 202-206, PO Box 6800, Bridgewater, NJ 08807-0800, USA. [3]Department of Genetics and Development, Columbia University, New York, NY 10032, USA. [4]Nina Ireland Laboratory of Developmental Neurobiology, University of California San Francisco, Genetics and Development, San Francisco, CA 94158-2611, USA. [5]Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, 675 Hoes Lane, Piscataway, NJ 08854, USA. [6]Department of Cell and Molecular Biology, Institute of Biochemistry and Biotechnology, Technical University of Braunschweig, 38106 Braunschweig, Germany. [7]Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032, USA.

*Present address: UBE, Nobels väg15A, Karolinska Institutet, S-17177 Stockholm, Sweden
†These authors contributed equally to this work
‡Author for correspondence (johan.ericson@ki.se)

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acts at long range in vivo (Briscoe et al., 2001) and a graded distribution of the Shh ligand has been demonstrated (Chamberlain et al., 2008). Together with genetic analyses of Gli genes (Ding et al., 1998; Matise et al., 1998; Persson et al., 2002) and gain-of-function studies in chick (Stamataki et al., 2005), these data are consistent with a model in which the extracellular Shh gradient is translated into an intracellular Gli activator-to-repressor gradient along the ventral-to-dorsal axis of the neural tube (Dessaud et al., 2008). Consistent with this view, ventral subtype identities are lost in mice that lack Shh, as well as in Smo mutants that are unable to transduce the Shh signal (Chiang et al., 1996; Wijgerde et al., 2002). However, ventral patterning is partly restored in *Shh;Gli3* and *Smo;Gli3* compound mutants, despite the abolition of graded Shh signaling activity (Litingtung and Chiang, 2000; Wijgerde et al., 2002). The underlying reason for this rescue of ventral cell types remains unclear, but these data reveal the presence of mechanisms that can diversify ventral cell identities in a manner independent of a functional Shh gradient. Moreover, genetic analyses of Gli genes suggest a conjoined induction of FP cells and p3 progenitors in the ventral neural tube, as the generation or absence of one population is coincident with that of the other (Ding et al., 1998; Matise et al., 1998; Bai et al., 2004; Lei et al., 2004). This raises the possibility that non-graded mechanisms of Shh signaling may contribute to the spatial patterning of these cell types.

Previous studies indicate that neural cells interpret both the concentration and duration of Shh signaling (Roelink et al., 1995; Ericson et al., 1997; Dessaud et al., 2007), implying that neural progenitors are endowed with a capacity to integrate Shh signaling and buffer Shh responses. Recent data indicate that negative feedback inhibition of the Shh pathway by Ptc1 contributes to this process (Dessaud et al., 2007), consistent with the facts that Ptc1 interferes with signal transduction by Smo and is markedly upregulated in cells in response to Shh (Goodrich et al., 1996; Marigo and Tabin, 1996; Goodrich et al., 1999). However, cells in the intermediate neural tube appear less competent to respond to Shh when compared with those found at dorsal and ventral extremes (Ruiz i Altaba et al., 1995). As induction of Ptc1 in the neural tube proceeds in a ventral-to-dorsal fashion, this observation indicates that the activity of Ptc1 alone is insufficient to explain the low Shh responsiveness in the intermediate neural tube. BMP and Wnt signals emanating from the dorsal neural tube have been implicated in opposing graded Shh signaling (Ulloa and Martí, 2010), but their actions are also unlikely to explain these differences because dorsal progenitors are exposed to the highest concentrations of these signals. The expression of HD proteins has been used as the primary readout of graded Shh signaling in neural tissue, but it is notable that their expression is also correlated with cells that exhibit differing potential to respond to Shh (Ruiz i Altaba et al., 1995; Ericson et al., 1997). Recent data further indicate a dynamic regulation of these transcription factors over the period in which ventral pattern is established (Dessaud et al., 2007; Chamberlain et al., 2008). This raises the possibility that these transcriptional response genes do not operate solely downstream of the Shh pathway, but rather that their activities are directly integrated with mechanisms of cellular interpretation of the Shh morphogen.

In this study, we address the role of HD proteins in morphogen interpretation, providing evidence that Pax6, Nkx2.2 and Nkx2.9 modulate the strength of Shh signaling in neural progenitor cells. Our analysis reveals a dual role for Nkx2 proteins in the induction of FP cells and p3 progenitors, through cell-autonomous feed-forward amplification of Shh signaling. By contrast, Pax6, which is expressed dorsal to Nkx2.2^{+} progenitors, functions as a suppressor of ventral cell fates through its ability to dampen the impact of Shh signaling. The feedback activity exerted by HD proteins provides an intrinsic, non-graded regulation of Gli activity in ventral progenitors, and is necessary for establishment of a robust patterning output in response to graded Shh signaling. Our study further reveals that the spatial patterning of FP cells and p3 progenitors is likely to be determined by a temporal shift in neuronal potential, rather than by exposure of cells to different concentrations of Shh. These data establish that progressive intrinsic changes within the field of responding cells have a profound influence on Shh morphogen response, elucidating a mechanism that translates graded Shh signaling into a stepwise response that renders cells less sensitive to fluctuations of the Shh gradient over time.

MATERIALS AND METHODS

DNA constructs

pCAGGS constructs of Nkx2.2, Nkx2.9, Pax6, Nkx2.2 HD-EnR, Nkx2.2HD-Vp16, Nkx2.2ΔTN and Pax6PD-EnR (Muhr et al., 2001); Ptc$^{\Delta loop2}$ (Briscoe et al., 2001); Gli3R and EGFP (Persson et al., 2002); and Nkx2.2 in RCASBPB vectors (Nkx2.2^{LATE}) (Briscoe et al., 2000) were used. FoxA2 was provided by D. Epstein (University of Pennsylvania, PA, USA) and Ngn2 was isolated from mouse cDNA library.

In ovo electroporation

Different cDNAs were electroporated either alone or in combination according to the text, into Hamburger and Hamilton (HH) stage 10 chick embryos (Briscoe et al., 2000), which were incubated for the indicated time periods and then processed for immunohistochemistry and in situ hybridization histochemistry at different time points. In co-electroporation experiments, the constructs were mixed at a ratio of 1:1, with the exception of the eGFP vector that was mixed at a 1:2 ratio and Nkx2.2+Ngn2/3 or Nkx2.2+Ptc$^{\Delta loop2}$ at a 1:5 ratio.

Neural explant culture

Neural tubes were electroporated in ovo with Pax6 or GFP vectors and neural plate explants were subsequently isolated essentially as described (Yamada et al., 1993). Explants were cultured for 24 hours in the presence of 4 nM Shh-N and processed for analysis (Ericson et al., 1996).

Immunohistochemistry and in situ hybridization

Immunohistochemical localization of proteins was performed as described (Yamada et al., 1993). Antibodies used: mouse 9E10, Shh, Nkx2.2, FoxA2 and Pax6 (Developmental Studies Hybridoma Bank); goat β-galactosidase (Biogenesis); rabbit GFP (Molecular Probes), Sox1and Ngn2 (Bylund et al., 2003); and guinea pig Olig2 (Novitch et al., 2001), Isl1/2 and Nkx2.9 (Briscoe et al., 2000). Images were collected on a Zeiss LSM510 confocal microscope.

In situ hybridization was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993) using chick probes for *Shh* (Marigo and Tabin., 1996), *Sim1* (Briscoe et al., 2000), *Nkx6.1*, *Dbx2* (Vallstedt et al., 2001), *Gli3* (C. Tabin) and *FoxJ1* isolated from chick cDNA library; and mouse *Gli3* (Persson et al., 2002).

Mutant mouse strains

Nkx2.2 (Briscoe et al., 1999), Nkx2.9 (Pabst et al., 2003) and Pax6 (St-Onge et al., 1997) mutant mice have been previously described. Pax6 mutant mice were provided by Peter Gruss.

RESULTS

Nkx2 proteins are required for specification of FP cells

To explore early patterning of the ventral-most neural tube, we first analyzed the spatiotemporal expression profile of Shh-regulated transcription factors that mark distinct ventral progenitor domains

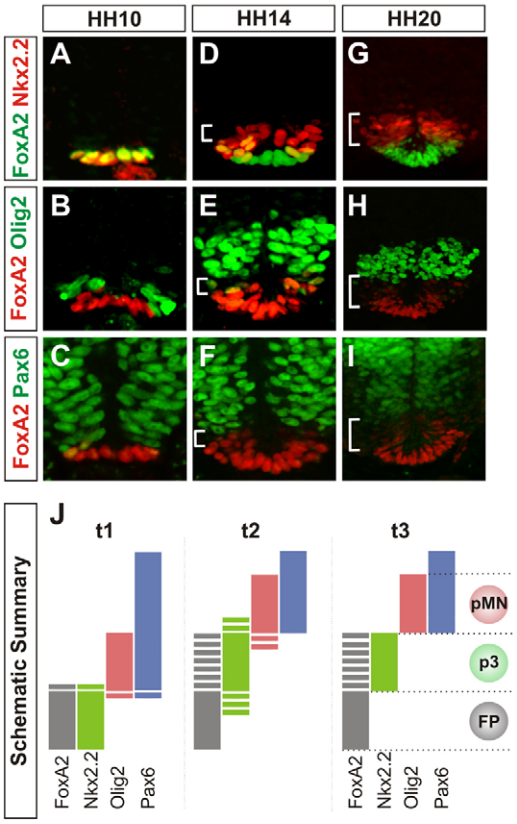

Fig. 1. Temporal expression profile of Nkx2.2 in the ventral neural tube. (**A-I**) Temporal expression analysis of Nkx2.2 and FoxA2 (A,D,G), FoxA2 and Olig2 (B,E,H), FoxA2 and Pax6 (C,F,I) on transverse sections of caudal neural tube at HH10 (A-C), HH14 (D-F) and HH20 (G-I). (**J**) The progressive establishment of ventral progenitor domains (not to scale). t, time point.

in the developing chick neural tube. By the time defined progenitor domains have been established, the HD protein Nkx2.2 is expressed selectively in p3 progenitors that generate V3 neurons dorsal to the floor plate (FP) and ventral to the motor neuron progenitor (pMN) domain (Fig. 1G,H) (Briscoe et al., 1999). But at earlier stages, soon after neural tube closure, Nkx2.2 was co-expressed with the FP determinant FoxA2 (Sasaki and Hogan, 1994) in presumptive FP cells (Fig. 1A). No cells exhibited a transcriptional p3 progenitor identity at early stages: cells flanking the ventral midline domain instead exhibited characteristics of MN progenitors, defined by their expression of the bHLH protein Olig2 and HD proteins Pax6 and Nkx6.1 (Fig. 1B,C; data not shown) (Ericson et al., 1997; Briscoe et al., 2000; Novitch et al., 2001). The expression pattern of Nkx2.2 was dynamic, expanding progressively dorsally while concomitantly becoming downregulated in differentiating FoxA2$^+$/Shh$^+$ FP cells at the ventral midline (Fig. 1A,D,G; data not shown). Expansion of the Nkx2.2$^+$ domain correlated with a spatial separation of the FP and pMN domains through the progressive establishment of an intervening Nkx2.2$^+$/FoxA2$^-$/Olig2$^-$ p3-progenitor domain between these cell groups (Fig. 1D-I). Nkx2.2 is a known repressor of Pax6 and Olig2, and is therefore likely to underlie the suppression of these transcription factors in cells fated to become p3 progenitors (Fig. 1J) (Briscoe et al., 2000; Muhr et al., 2001; Novitch et al., 2001).

We considered whether the transient expression of Nkx2.2 in ventral midline cells is indicative of an early and unappreciated role for Nkx2.2 in FP specification. To examine this, we monitored ventral patterning in compound mutant mice (termed Nkx2 mutants) lacking Nkx2.2 (Sussel et al., 1998) and the functionally related protein Nkx2.9 (Pabst et al., 2003) (Fig. 2G,H). The progressive establishment of ventral progenitor domains in wild-type mice was similar to that observed in chick embryos, apart from a more prominent expression of FoxA2 in the mouse p3 domain (Fig. 1A-I; Fig. 2A-F). In Nkx2.2 single mutants, there is a reduction of V3 neurons but the FP appears intact (Briscoe et al., 1999), whereas loss of Nkx2.9 does not affect FP cells or V3 neurons (Pabst et al., 2003). Strikingly, in Nkx2 compound mutants the number of FoxA2$^+$/Shh$^+$ FP cells was drastically reduced by ~80% in E10.5 spinal cord (Fig. 2K,L) together with a complete loss of *Sim1*$^+$ V3 neurons (Fig. 2I,J). This loss of both FP cells and V3 neurons was accompanied by a pronounced ventral expansion of Olig2 expression and by overproduction of MNs, as revealed by Isl1/2, Hb9 and peripherin expression (Fig. 2M-P; data not shown). The Nkx2.9 locus of Nkx2 mutants harbors a LacZ transgene, and ventral Olig2$^+$ cells in these mutants scored positive for LacZ, providing direct evidence for conversion of presumptive FP cells and p3 progenitors into MN progenitors (Fig. 2Q-T). Moreover, residual FoxA2$^+$ cells often co-expressed Olig2, indicating that a subset FP cells in Nkx2 mutants expressed a mixed cellular identity not apparent in controls (Fig. 2M,N). The early reduction of FP cells further resulted in functional impairment of FP properties, including guidance of commissural axons in the spinal cord (H.-H.A., personal communication). These data reveal an unanticipated dual requirement for Nkx2 proteins in establishment of FP and p3-progenitor fates in the ventral neural tube (Fig. 2U).

A sequential role for Nkx2 proteins in induction of FP cells and V3 neurons

We next assessed whether Nkx2.2 is sufficient to induce ectopic FP differentiation in the neural tube. Nkx2.2 is sufficient to induce V3 neurons but an early role of Nkx2.2 in FP specification may not have been revealed in previous studies (Briscoe et al., 2000) as the retroviral expression vector used to transduce Nkx2.2 has a significant delay in the initiation of gene expression. To accelerate this process, we expressed Nkx2.2 under control of a hybrid β-actin-CMV promoter (termed Nkx2.2^{EARLY}) that drives rapid gene expression in neural progenitors. We then compared the ability of Nkx2.2^{EARLY} to induce FP cells with that of Nkx2.2 expressed from retroviral vectors (termed here Nkx2.2^{LATE}) by in ovo electroporation of HH stage 10 chick embryos.

In experiments with Nkx2.2^{LATE}, expression of Nkx2.2 was initiated ~15-18 hours post transfection (hpt) and resulted in ectopic induction of the V3 neuron marker *Sim1* by 48 hpt (Fig. 3B,C; data not shown). No ectopic induction of the FP marker *Shh* was detected in these experiments (Fig. 3D). By contrast, in experiments with Nkx2.2^{EARLY}, Nkx2.2 was detectable within 3 hpt and extensive induction of ectopic FoxA2 and *Shh* expression was evident by 20 hpt and 30 hpt, respectively (Fig. 3E,F,H; data not shown). Furthermore, induction of FoxA2 and suppression of Pax6 expression and that of the HMG protein Sox1 was already observed at 10 hpt (see Fig. S1A-C in the supplementary material; data not shown). As Sox1 is expressed by neuronal progenitors but not FP cells (Charrier et al., 2002) and we observe a robust expression of the FP markers Shh and

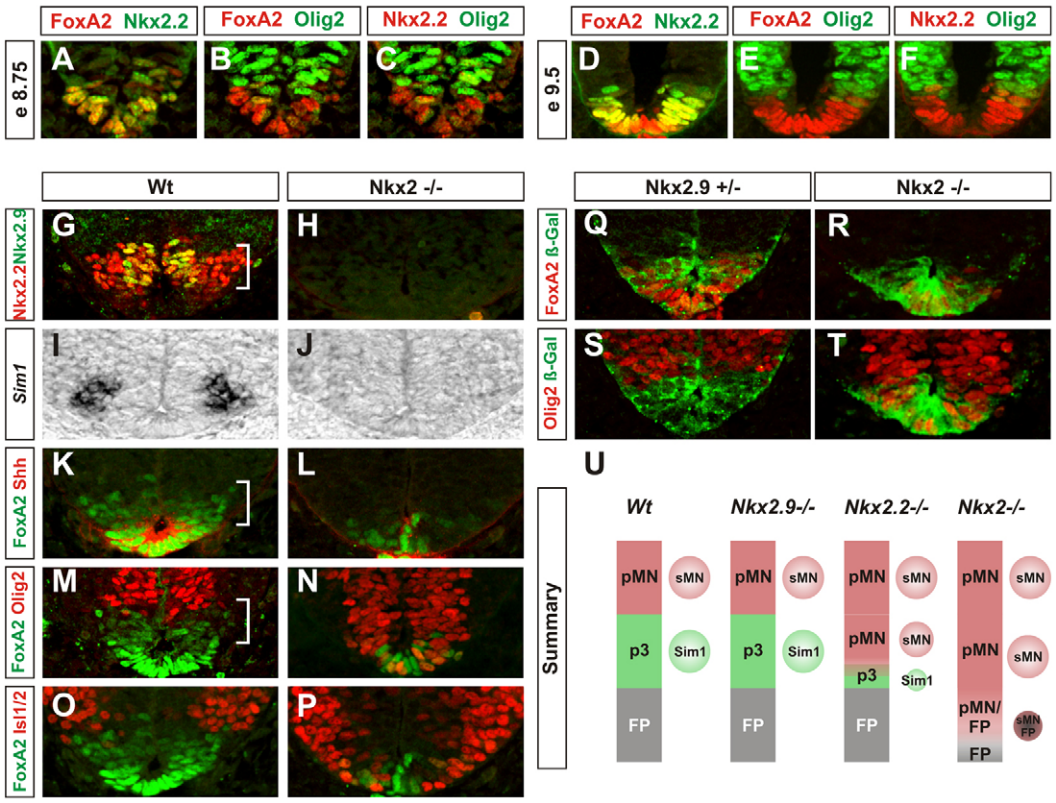

Fig. 2. Loss of V3 neurons and FP cells, and corresponding gain of motoneurons in *Nkx2.2;Nkx2.9* compound mutant mice. (**A-F**) Temporal expression analysis at embryonic day (E)8.75 (A-C) and E9.5 (D-F) showing the temporal generation of FP and p3 domain at spinal cord levels. (**G-P**) E10.5 embryos lacking Nkx2.2 and Nkx2.9 (Nkx2 mutants; G,H) show a loss of *Sim1*$^+$ V3 neurons (I,J) and reduction of FoxA2$^+$/Shh$^+$ FP cells (K,L), accompanied by a ventral expansion of the pMN domain determinant Olig2 (M,N) and concomitant overproduction of Isl1$^+$ motoneurons (O,P). (**Q-T**) β-Gal expressed from the Nkx2.9 locus colocalizes with FoxA2 (Q) but not with Olig2 (S) in wild-type mice but is expressed in more ventral Olig2$^+$ cells in Nkx2 mutants (T). Residual FoxA2$^+$ cells in Nkx2 mutants express β-gal (R). (**U**) Changes in FP and ventral progenitor domains (p3, pMN) and corresponding cell fates in wild type, Nkx2.9, Nkx2.2 and Nkx2 mutants.

FoxJ1(Yu et al., 2008) by 40 hpt (see Fig. S1D,E in the supplementary material), these data indicate that FoxA2$^+$/Shh$^+$ cells induced by Nkx2.2 acquire definitive FP character. Importantly, induction of FoxA2 and extinction of Sox1 by Nkx2.2 occurred well before initiation of *Shh* expression in ectopic FP cells (Fig. 3E-H; see Fig. S1A-C in the supplementary material). Analogous results were seen in similar experiments with Nkx2.9 (Fig. 3M). Thus, Nkx2 proteins are sufficient to induce FP differentiation at dorsal positions of the neural tube, and this activity appears to be limited to early stages of neural development (Fig. 3A; see below).

Nkx2.2 can function as both a transcriptional activator and repressor (Watada et al., 2000; Muhr et al., 2001). To determine how Nkx2.2 specifies FP fate, we examined whether activator (Nkx2.2HD-VP16) or repressor (Nkx2.2HD-EnR) forms of Nkx2.2 (Muhr et al., 2001) could mimic the FP-inducing activity of Nkx2.2. Whereas Nkx2.2HD-VP16 was unable to induce ectopic FoxA2 or *Shh* expression (Fig. 3M; data not shown), Nkx2.2HD-EnR mimicked the activity of Nkx2.2 by inducing ectopic FP differentiation (Fig. 3I-M). A variant of Nkx2.2 that cannot effectively recruit Gro/TLE co-repressors (Nkx2.2ΔTN) (Muhr et al., 2001) also failed to induce FP cells (Fig. 3M). Consistent with the transient expression of Nkx2.2 in the FP lineage, expression of Nkx2.2HD-EnR also induced endogenous Nkx2.2 transcription (data not shown), so we cannot exclude that, once induced, Nkx2.2 could provide activities in addition to transcriptional repression. Nevertheless, initial activation of FoxA2 expression and FP differentiation is triggered by Nkx2.2-mediated transcriptional repression.

Nkx2 proteins intrinsically amplify Shh signaling in neural progenitors

FoxA2 is a direct target of Shh signaling (Sasaki et al., 1997) and is both required and sufficient for specification of FP identity (Ang and Rossant, 1994; Sasaki and Hogan, 1994; Weinstein et al., 1994), raising the key issue of how Nkx2.2-mediated repression induces FoxA2 in the dorsal neural tube where the ambient Shh concentration is low. One possibility would be that Nkx2.2 operates not only downstream of the Shh pathway, but also directly influences interpretation of the Shh gradient by modulating the intrinsic strength of Shh signaling. To test this, we monitored overall Gli-transcriptional activity using a Shh-regulated Nkx2.2 cis-regulatory module (CRM-Nkx2.2) similar to that previously described (Lei el al., 2006). In vivo expression of CRM-Nkx2.2-GFP produced GFP activity confined to the p3 domain (Fig. 4A). In response to forced Nkx2.2 expression, CRM-Nkx2.2 became broadly activated in the neural tube (Fig. 4A,B). CRM-Nkx2.2 contains a single functional Gli-binding site (GBS) (Lei et al., 2006) and, importantly, inactivation of this GBS abolished dorsal activation of CRM-Nkx2.2 by Nkx2.2 (Fig. 4A-D). Thus, forced expression of Nkx2.2 results in Gli-dependent activation of CRM-

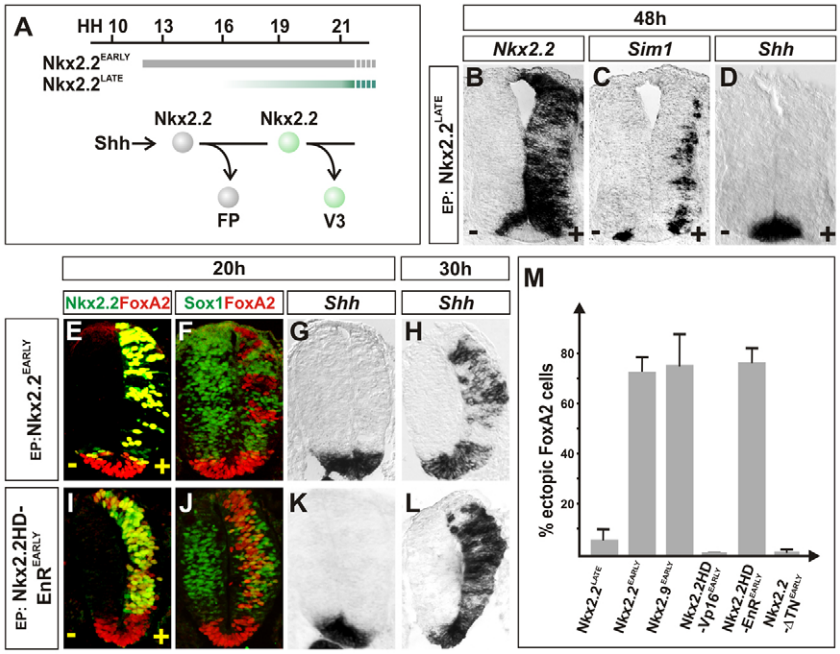

Fig. 3. Sequential induction of FP cells and V3 neurons by the repressor Nkx2.2. (**A**) Nkx2.2 induces FP at early stages, and later V3 neurons. (**B-D**) Forced expression of Nkx2.2^{LATE} at HH10 (B) results in ectopic induction of the V3 neuronal marker *Sim1* 48 hpt (C), but not *Shh* (D). (**E-H**) By contrast, forced expression of Nkx2.2^{EARLY} induces FoxA2 (E,F) and suppresses Sox1 (F) by 20 hpt. Induction of *Shh* is not observed at 20 hpt (G), but is extensive by 30 hpt (H). (**I-L**) Nkx2.2HD-EnR^{EARLY} mimics the ability of full-length Nkx2.2 to induce FP (compare with E-H). (**M**) Quantification of the FoxA2-inducing capacity of different Nkx2 protein variants. Values are presented as mean ± s.d. EP, electroporation.

Nkx2.2 at dorsal positions. Interestingly, the increase in net GliA activity in response to Nkx2.2 was correlated with pronounced downregulation of *Gli3* expression (Fig. 4E), a major suppressor of Shh-target genes in the neural tube (Litingtung and Chiang, 2000; Persson et al., 2002). Co-expression of Nkx2.2 with an obligate Gli3 repressor (Gli3R) (Persson et al., 2002) suppressed FP induction (Fig. 4E,F,H), indicating that reduction of Gli3R levels is a prerequisite for Nkx2.2-mediated FP induction. These data establish that Nkx2.2 functions to intrinsically amplify Shh-Gli signaling, and suggest that a key role for Nkx2.2 in this process is to reduce GliR levels by downregulating *Gli3* expression, thereby increasing net levels of GliA in these cells.

To examine whether FP induction by Nkx2.2 requires ongoing signaling, we took advantage of a dominant-negative form of Ptc, termed Ptc$^{\Delta loop2}$ (Briscoe et al., 2001), which blocks Shh signal transduction through dominant inhibition of Smo. Expression of Ptc$^{\Delta loop2}$ with Nkx2.2 resulted in a drastic reduction of ectopic FoxA2 expression and FP differentiation in the neural tube (Fig. 4G,H; data not shown), indicating that active Shh signaling is required for Nkx2.2-mediated FP induction. By contrast, Nkx2.2 was still able to repress Pax6 and *Gli3* expression under these conditions (Fig. 4G; see Fig. S2 in the supplementary material). Thus, the FP-inducing capacity of Nkx2.2 can be separated from its role as a repressor of Pax6 (Fig. 4E) (Briscoe et al., 2000). This finding argues against the possibility that inhibition of FP induction under conditions of blocked Shh responses merely reflects sustained Pax6 and *Gli3* expression in neural progenitors (see below).

A shift in neuronal potential underlies spatial patterning of FP cells and V3 neurons

The FP and p3 domains are established sequentially and the dual requirement for Nkx2 proteins in this process raises the issue of whether these cells are induced by a similar concentration of Shh. Genes conferring neuronal potential are sparsely expressed at the time of FP induction (Sommer et al., 1996) (J.E., unpublished) and differentiated FP cells express glial-like traits rather than neuronal properties (Placzek et al., 1993). We therefore considered whether a 'non-neuronal-to-neuronal' switch in progenitor potential could account for this activity. Proneural bHLH transcription factors are central mediators of neurogenesis (Bertrand et al., 2002), and the p3 domain expresses the bHLH genes Ngn3 and Ascl1 (Briscoe et al., 1999) (J.E., unpublished). Consistent with a temporal switch from glial to neuronal competence, early expression of Ngn2, Ngn3 and Ascl1 each suppressed the ability of Nkx2.2 to induce ectopic FP differentiation (Fig. 5A-D; data not shown). Moreover, expression of these bHLH factors at the ventral midline repressed FoxA2 and resulted in ectopic and premature generation of *Sim1*$^{+}$ V3 neurons within the FP domain without significantly affecting *Shh* expression (Fig. 5E-K; data not shown). These data support the view that the gradual emergence of neurogenic factors underlies the glial-to-neuronal switch in fate determination of Nkx2.2^{+} progenitors in the ventralmost neural tube (Fig. 5L).

Pax6 suppresses FP fate by opposing Shh signaling activity

The identification of Nkx2.2 as a sensitizer of Shh responsiveness raises the issue of whether other patterning HD proteins contribute to regulation of Shh responsiveness. Pax6 suppresses Nkx2.2 expression and high levels of Pax6 are correlated with a low probability of cells adopting FP fate in response to Shh (Ruiz i Altaba et al., 1995; Ericson et al., 1997). These observations raise the possibility that Pax6 counteracts Shh responses in neural progenitors. Consistent with this idea, we found that forced expression of Pax6 in the ventral neural tube suppressed FoxA2 and *Shh* expression (Fig. 6A-D) and upregulated *Gli3* transcription (Fig. 6E,F). Pax6 also suppressed FoxA2 induction in Pax6-transfected naïve neural plate explants exposed to 4 nM Shh (Fig. 6G,H), a Shh concentration otherwise sufficient to induce FP differentiation in vitro (Roelink et al., 1995; Ericson et al., 1996). The activity of Pax6 was cell autonomous as non-transfected Pax6^{-} cells retained the capacity to adopt FP identity in response to this concentration of Shh (Fig. 6H). As Pax6 represses *Nkx2.2* indirectly via transcriptional activation (Muhr et al., 2001), we tested whether suppression of FP differentiation by Pax6 is also indirect, through transcriptional activation. We analyzed FP induction and *Gli3* expression in response to neural expression of

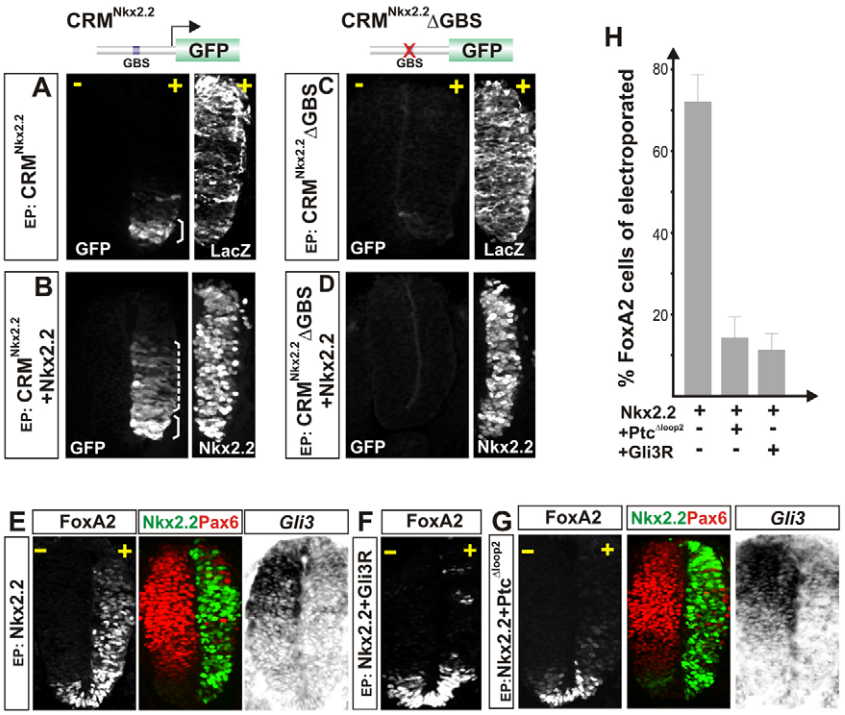

Fig. 4. Nkx2.2 is a cell-intrinsic sensitizer of Shh responses. (**A-D**) GFP reporter expressed from an Nkx2.2 cis-regulatory module (CRM-Nkx2.2-GFP) recapitulates expression of Nkx2.2 in vivo at 20 hpt (A). Misexpression of CRM-Nkx2.2-GFP together with Nkx2.2 results in graded dorsal expansion of reporter expression (B, dashed bracket), reflecting the oncoming gradient of GliA in the neural tube. This effect requires a functional GBS in the Nkx2.2 CRM (C,D). Hsp70-LacZ is used as an internal electroporation control. (**E-H**) Expression of Nkx2.2 in the neural tube induces FoxA2 expression, concomitant with repression of Pax6 and *Gli3* (E,H). Misexpression of Gli3R abrogates the ability of Nkx2.2 to induce FoxA2 but not to repress Pax6 (F,H; data not shown). Co-expression of Ptc$^{\Delta loop2}$ with Nkx2.2 drastically reduces the ability of Nkx2.2 to induce FoxA2 but not to repress Pax6 or *Gli3* (G,H).

a reverse-function Pax6 repressor (Pax6PD-EnR) (Muhr et al., 2001). Strikingly, expression of Pax6PD-EnR extinguished *Gli3* expression and induced FP differentiation, as indicated by loss of Sox1 expression and ectopic induction of FoxA2 and *Shh* (Fig. 6I-L). Induction of Nkx2.2 and repression of endogenous Pax6 expression were also observed in these experiments (data not shown) (Muhr et al., 2001). We next performed epistasis experiments in which we co-electroporated Nkx2.2 and Pax6 at different concentration ratios, and observed that Pax6 abrogated the effects of Nkx2.2 activity on FoxA2 and *Gli3* expression in a dose-dependent manner (see Fig. S3A-F in the supplementary material), demonstrating that regulation of *Gli3* expression by Nkx2.2 and Pax6 is independent of their cross-repressive interactions. Consistently, Pax6 also opposed ventral patterning when overexpressed within the endogenous Pax6 domain, as indicated by ventral expansion of *Dbx2* expression and reduced *Nkx6.1* expression (Fig. 6M-O) (Briscoe et al., 2000). These data imply that Pax6 is sufficient to suppress FP induction and indicate that levels of Pax6 expression play a general role in counteracting Shh-responses in the ventral neural tube by increasing the net ratio of GliR to GliA present in these cells.

Although Pax6 is sufficient to upregulate *Gli3* transcription, analysis of Pax6 mutant mice indicates that Pax6 is not absolutely required for neural *Gli3* expression (Fig. 6R,T) (St-Onge et al., 1997). However, at E10.5 there was a significant reduction of *Gli3* expression in ventral progenitors of Pax6 mutants, coinciding precisely with the dorsal expansion of Nkx2.2^{+} p3 progenitors (Fig. 6Q-T). Despite this, there was no extensive expansion of the FP domain in Pax6 mutants (Fig. 6U,V) (Ericson et al., 1997), possibly reflecting the limited time frame in which neural progenitors are competent to acquire FP identity in response to high concentrations of Shh.

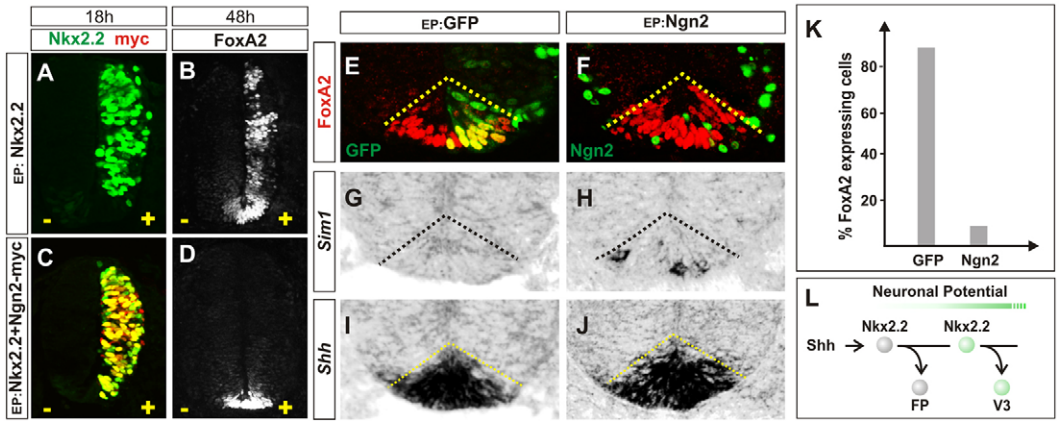

Fig. 5. Proneural bHLH proteins suppress FP differentiation and promote Nkx2.2-mediated induction of V3 neurons. (**A-D**) The ability of Nkx2.2 to induce FoxA2 (A,B) is suppressed when co-expressed with proneural bHLH protein Ngn2 (C,D). (**E-K**) Misexpression of Ngn2 in only a few FP cells (F,K) suppresses FoxA2 expression (E,F) and induces *Sim1*$^{+}$ cells (G,H). No detectable effect on the overall expression of *Shh* is observed (I,J). (**L**) Changes in progenitor potential and how they enable the dual role of Nkx2.2 in specification of FP cells and V3 neurons.

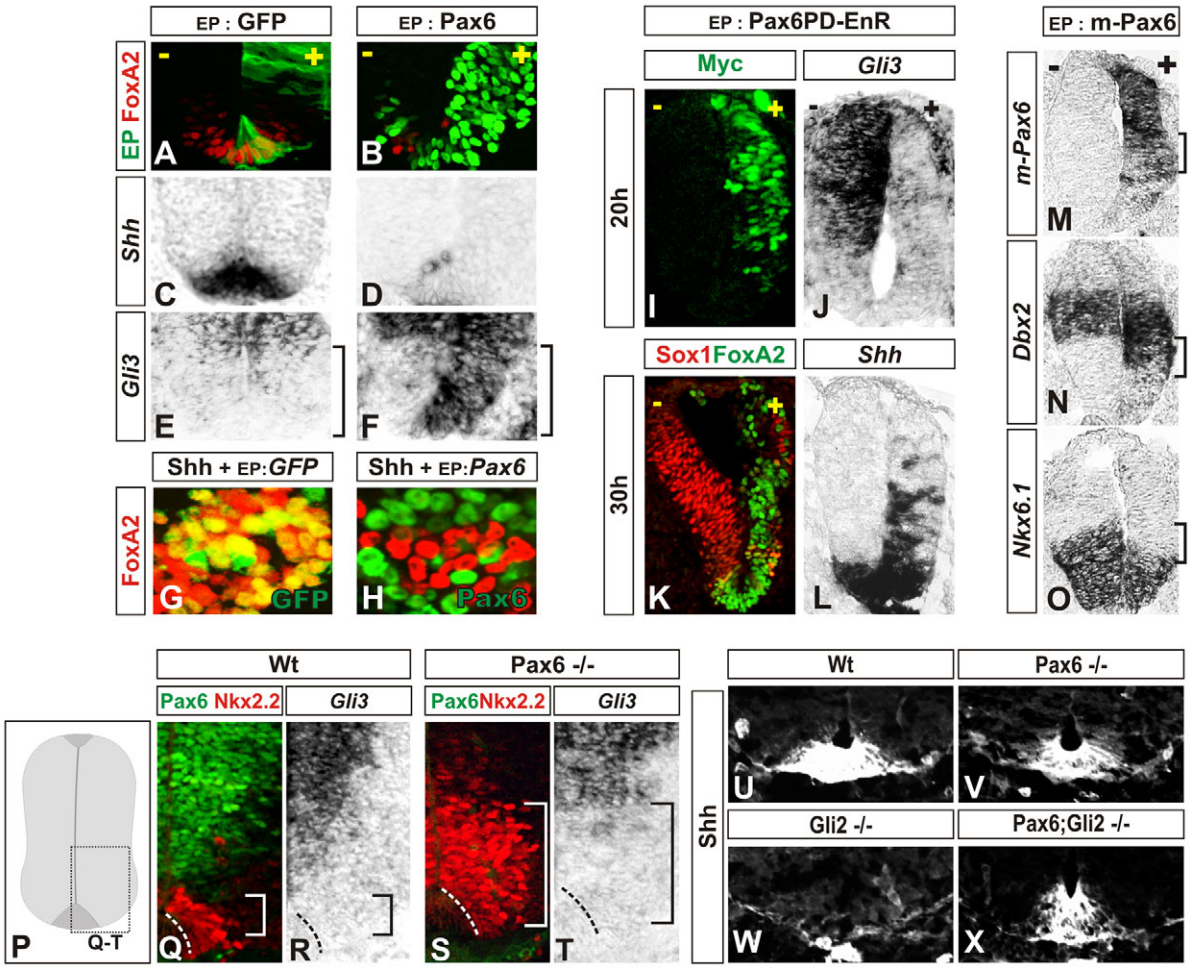

Fig. 6. Pax6 stabilizes the Gli repressor gradient and suppresses FP differentiation. (**A-F**) Misexpression of Pax6 (B,D,F) but not GFP (A,C,E) represses FoxA2 (A,B) and *Shh* expression (C,D) within the endogenous FP. Ventral upregulation of *Gli3* was observed only with Pax6 (E,F). (**G**,**H**) Misexpression of Pax6 blocks the Shh-mediated induction of FP differentiation in naïve neural plate explants electroporated with Pax6 or GFP and cultured in the presence of 4 nM Shh. In controls, most GFP-expressing cells co-express FoxA2 (G) and Shh (data not shown) after 24 hours in culture. By contrast, essentially no Pax6$^+$ cells express FoxA2 (H) or *Shh* (data not shown). (**I-L**) Pax6PD-EnR(myc) (I) represses *Gli3* (J) and results in ectopic induction of the FP marker FoxA2 (K) and *Shh* (L) after 30 hpt. A marked downregulation of Sox1 expression was also observed in these experiments (K). (**M-O**) Overexpression of mouse-Pax6 in the intermediate neural tube alters patterning, with ventral expansion of *Dbx2* (N) and restriction of *Nkx6.1* (O). (**P**) Schematic representation of the neural tube indicating the region analyzed in Q-T. (**Q-T**) Expression of Nkx2.2 and Pax6 in the caudal hindbrains of E10.5 wild-type (Q) and *Sey/Sey* (Pax6 mutant) embryos (S). In the absence of Pax6, there is ~3-fold dorsal expansion of the p3 domain (S). The ventral limit of *Gli3* expression (brackets in R,T) is retracted in Pax6 mutants (T), correlating with the dorsal expansion of Nkx2.2 expression. Broken lines indicate the boundary between the FP and the dorsally abutting Nkx2.2$^+$-p3 domain. (**U-X**) Expression of Shh in E10.5 embryos from wild-type (U), Pax6 (V), Gli2 (W) and *Pax6;Gli2* compound (X) mutants. Loss of Pax6 rescues the loss of Shh seen in Gli2 mutants (W,X).

Based on these observations, we examined whether Pax6 contributes to the suppression of FP differentiation observed in Gli2 mutants (Matise et al., 1998). Strikingly, when compared with a complete loss of FP cells in Gli2 single mutants (Fig. 6W), Shh$^+$ FP cells were restored at the ventral midline of the neural tube in *Pax6;Gli2* compound mutants (Fig. 6X). This finding provides genetic evidence that Pax6 suppresses FP fate by counteracting the Shh pathway.

DISCUSSION

Studies of Shh morphogen activity in the vertebrate central nervous system have focused on its extracellular concentration gradient and characterization of components of the canonical signal transduction pathway. By contrast, little is known about how changes in responding cells influence patterning output. We provide evidence that HD proteins, which are themselves subject to Shh regulation, exert cell-intrinsic feedback modulation on the Shh pathway that contributes to normal patterning of the ventral neural tube. Nkx2 proteins are induced by Shh and function to amplify Shh responses, whereas Pax6 antagonizes Shh output by elevating the level of GliR. Our data also indicate that spatial patterning of FP cells and p3 progenitors is determined by a temporal switch in neuronal progenitor potential, rather than by exposure of cells to different Shh concentrations. These data demonstrate that dynamic, non-graded modulation of signaling strength and temporal alterations of cellular competence are essential for interpretation of Shh signaling, and suggest a model in which the Shh pathway and HD proteins are integrated in an intimate transcriptional feedback circuit (Fig. 7A,B).

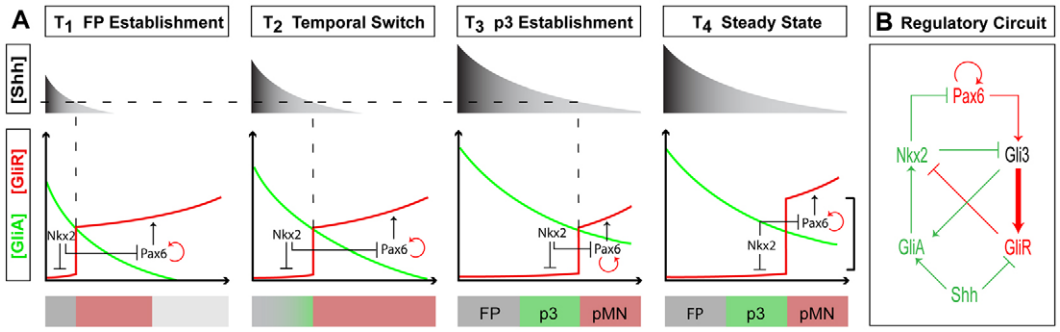

Fig. 7. A non-graded model of Shh morphogen interpretation. (**A**) Nkx2 proteins and Pax6 have opposing effects on total GliR levels that serve to buffer Gli output of Shh signaling, providing a robust non-graded response. (T_1) As the Shh gradient is established, Nkx2 proteins reduce total GliR levels and repress Pax6, initially to induce FP, while Pax6 maintains total GliR levels above an Nkx2.2-inducing threshold at more dorsal positions (broken lines). (T_2) A temporal gating of Nkx2 protein activity by a switch to neuronal competence leads to initiation of p3 domain induction as Nkx2.2 expression continues to expand dorsally. This limits the size of the FP. Thus, the threshold of Shh signaling for induction of these fates is likely to be similar (broken lines). (T_3) The p3 domain reaches its maximum extent as the inductive capacity of the Shh gradient becomes limiting. (T_4) At the steady state, Pax6 reinforces the rate of resistance to Shh signaling in the pMN domain, and suppression of GliR levels and Pax6 by Nkx2 proteins maintains the p3 domain. (**B**) The regulatory circuit depicting modulatory feedback loops between Nkx2, Pax6 and Gli transcription factors that govern induction of ventral progenitor fates and maintenance of sharp boundaries between the domains. Expression of *Gli3* gives rise to a higher stoichiometric ratio of GliR to GliA. Green indicates steps required for amplification of Shh signaling, while red denotes mechanisms of intrinsic resistance. For simplicity, feedback inhibition by, for example, Ptc1 and other patterning transcription factors that may also contribute to the modulation of Shh responses (e.g. Olig2) have been omitted. We also do not exclude the possibility that Nkx2 and Pax6 proteins could also act via additional parallel mechanisms.

Spatial patterning of FP and p3 domains involves a temporal shift in progenitor cell potential

Our findings reveal an unexpected requirement for Nkx2 activity in induction of FP cells. This activity raises questions about how selection between FP and p3 progenitor fates is accomplished. FoxA2 is an established FP determinant (Ang and Rossant, 1994; Sasaki and Hogan, 1994), and could have possibly explained this specificity; however, like Nkx2.2, its expression is not exclusively confined to the FP lineage (Fig. 2) (Ruiz i Altaba, 1996; Ferri et al., 2007; Jacob et al., 2007), arguing that other mechanisms coordinate this process. Our data indicate that FP cells and p3 progenitors are induced by a similar concentration of Shh but at different times, and that their pattern of generation is determined by a non-neuronal-to-neuronal shift in progenitor potential. This idea is consistent with genetic studies of Gli genes, including the current analysis *Pax6;Gli2* compound mutants, that imply a conjoined induction of FP cells and p3 progenitors (Matise et al., 1998; Ding et al., 1998; Bai et al., 2004; Lei et al., 2004) (this study). Accordingly, at early stages when expression of Nkx2.2 is limited to the ventral midline, cells acquire FP identity; whereas more dorsal Nkx2.2$^+$ cells, induced after the neurogenic switch, acquire p3 progenitor cell properties. Considering the functional requirement for Nkx2.2 in FP specification at early stages, Nkx2.2 cannot be viewed as a devoted p3 progenitor determinant. Our model therefore differs from a recent study in which ventral midline cells were proposed to initially have a Nkx2.2$^+$ p3-like character and that implied that Shh signaling dynamics are the major determinant in the selection between FP and p3 fates (Ribes et al., 2010). Nevertheless, a switch to neuronal potential would explain not only the dual role of Nkx2.2, but also that of FoxA2 in the hindbrain, in which FoxA2 specifies FP fate and promotes the generation of 5HT neurons from Nkx2.2$^+$ progenitors (Jacob et al., 2007). As the FP itself secretes Shh, such a switch in competence would serve to delimit the extent of FP induction, preventing a continuous, or homogenetic, induction of FP cells throughout the neural tube. Dorsal induction of the roof plate by BMPs is likely to be constrained by the same mechanism (Liem et al., 1995). In addition, initiation of gliogenesis at late stages of neural development is associated with an overall loss of neuronal potential (Kessaris et al., 2001), a stage at which Nkx2.2 is implicated in specification of oligodendrocyte precursors rather than V3 neurons (Zhou et al., 2001). Collectively, these data indicate that the distinct output of specific cell fate-determining transcription factors depends on contextual changes in neuronal competence that accompany neural development.

Non-graded amplification of Shh signaling by Nkx2 proteins in neural progenitors

The dual role of Nkx2.2 in establishing the FP and p3 domains appears to involve cell-intrinsic amplification of Shh signaling strength in neural progenitors. Nkx2.2 is sufficient to induce FP differentiation at dorsal positions at which the ambient Shh concentration is low. Nkx2.2 therefore appears to augment Shh responses primarily by reducing the level of GliR in cells, both by suppression of Gli3 transcription and repression of Pax6, which counteracts Shh signaling (see below). These data, together with the restored induction of FP cells in *Pax6;Gli2* compound mutants, suggest that the level of GliA is not limiting per se in terms of transcriptional activation of Shh target genes. Instead, induction of FP fate and p3 progenitors is triggered when the level of GliA reaches a critical Nkx2.2-inducing threshold value, a value that is not absolute but rather depends on the status of counteracting GliR in responding cells. The onset of Nkx2.2 expression subsequently effects a transition from interpretation of the continuous Shh gradient to a discrete, stairway-like, response (Fig. 7A). In this process, Nkx2.2 concomitantly downregulates other transcription factors that promote more dorsal cell fates, including Pax6 and Olig2. Without this non-graded feed-forward activity of Nkx2 proteins, graded Shh signaling appears to be insufficient to pattern the ventral-most domains of the neural tube.

Pax6 provides responding cells with intrinsic resistance to Shh signaling

The establishment of ventral progenitor domains relies on cross-repressive interactions among Shh-regulated HD and bHLH proteins (Briscoe et al., 2000) and most transcription factors implicated in this process function as transcriptional repressors (Muhr et al., 2001; Novitch et al., 2001). The role of Pax6 has been more difficult to resolve, as Pax6 suppresses Nkx2.2 expression and FP fate only indirectly, through its function as a transcriptional activator (Fig. 6S) (Muhr et al., 2001). Tcf proteins and Groucho/TLE co-repressors have been implicated in repression of Nkx2.2 downstream of Pax6, but explain only partly the role of Pax6 in this process (Muhr et al., 2001; Lei et al., 2006). Nor can the repressor activity of Olig2 alone account for the activity of Pax6, as Olig2 is neither sufficient nor required to constrain expression of Nkx2.2 (Novitch et al., 2001; Zhou and Anderson, 2002). In fact, Olig2 may even prime cells for induction of Nkx2.2 by Shh, as induction of Olig2 precedes Nkx2.2 and Olig2 functions as a weak repressor of Pax6 within the pMN domain (Dessaud et al., 2007; Zhou and Anderson, 2002). Our data show that Pax6 opposes Shh signaling by upregulating the level of GliR, thereby counterbalancing the Shh gradient and ensuring that the net Gli activity is kept below an Nkx2.2-inducing threshold. This strategy obviates the need of a potent, dedicated repressor of Nkx2.2 in the pMN domain. The failure to stabilize high GliR levels can therefore explain the progressive dorsal expansion of Nkx2.2 in Pax6 mutants (Novitch et al., 2001), as well as the restored induction of FP differentiation observed in *Pax6;Gli2* compound mutants.

Loss of Pax6 function influences not only FP induction and the position of the p3/pMN boundary, but also results in more general patterning defects over time (Genethliou et al., 2009). Moreover, overexpression of Pax6 in the intermediate neural tube results in reduced *Nkx6.1* and ventral expansion of *Dbx2* expression. Pax6 appears therefore to be part of a more general gene regulatory circuit that provides cells with inherent resistance to respond to Shh signaling. Consequently, the positional establishment of the p3/pMN boundary that occurs in normal conditions and is impaired in Pax6 mutants should therefore delineate the approximate time at which the inductive capacity of the Shh gradient becomes limiting over the Pax6-regulated intrinsic resistance (Fig. 7). Such a role of Pax6 in neural patterning shows conceptual similarities to the action of the *Drosophila* repressor protein Brinker, which counteracts the morphogen signal Decapantaplegic (Dpp) to control the positional expression of target genes in the wing disc (Affolter and Basler, 2007).

Our findings suggest that it is possible to modulate patterning output by regulating Shh responsiveness in addition to the extracellular Shh gradient itself. This can be achieved as a function of the ground state of resistance set prior to signaling, but also by influencing the rate at which the resistance is reinforced over time. Like Pax6, Wnt signaling also opposes Shh signaling by upregulating *Gli3* transcription (Alvarez-Medina et al., 2008), and it is feasible that other extra-canonical pathways, or local transcriptional circuits, can influence Shh responses according to a similar logic. Intrinsic modulations of Shh responsiveness may therefore explain some local variations in growth and size of progenitor domains in the neural tube, including the extensive domain of FoxA2 expression in the midbrain in which Pax6 is not expressed (Gérard et al., 1995; Ferri et al., 2007). It is also feasible that Nkx2.1 influences Shh signaling in the ventral forebrain (Sussel et al., 1999), in a fashion similar to Nkx2.2 and Nkx2.9.

Importantly, our findings establish that Nkx2.2 and Pax6 are integral components of morphogen interpretation, and raise the possibility that the non-graded feedback regulation of signaling activity by transcription factors is a general strategy to achieve robust and non-graded responses to graded morphogen signals. The opposing modes of feedback on Shh signaling by Nkx2.2 and Pax6 render responding cells insensitive to fluctuations in the external environment and serve to stabilize the output of the Shh gradient (Fig. 7). Consequently, an $Nkx2.2^{+}$ progenitor will respond differently from a neighboring $Pax6^{+}$ cell, despite exposure to an identical Shh concentration. Furthermore, once induced, an $Nkx2.2^{+}$ cell is able to maintain its positional identity even under conditions in which the Shh concentration drops below the initial Nkx2.2-inducing threshold.

Studies of patterning during spinal cord (Ericson et al., 1996) and limb bud development (Harfe et al., 2004) have indicated that cells exposed transiently to Shh retain a memory of such exposure when challenged with a subsequent pulse of Shh. The activity of Nkx2.2 to autonomously amplify Shh signaling provides a mechanistic logic that plausibly underlies this phenomenon of 'memory' in neural tissue. Cellular memory may also result from inherent transcriptional changes in responding cells in the limb bud. Transcriptional feedback regulation of the Shh pathway, perhaps mediated by Hox genes (Chiang et al., 2001), could contribute to the establishment of anterior-posterior polarity in the limb bud.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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