Transduction of graded Hedgehog signaling by a combination of Gli2 and Gli3 activator functions in the developing spinal cord

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Summary

The three vertebrate Gli proteins play a central role in mediating Hedgehog (Hh)-dependent cell fate specification in the developing spinal cord; however, their individual contributions to this process have not been fully characterized. In this paper, we have addressed this issue by examining patterning in the spinal cord of *Gli2;Gli3* double mutant embryos, and in chick embryos transfected with dominant activator forms of Gli2 and Gli3. In double homozygotes, *Gli1* is also not expressed; thus, all Gli protein activities are absent in these mice. We show that Gli3 contributes activator functions to ventral neuronal patterning, and plays a redundant role with Gli2 in the generation of V3 interneurons. We also show that motoneurons and three classes of ventral neurons are generated in the ventral spinal cord in double mutants, but develop as intermingled rather than discrete populations. Finally, we provide evidence that Gli2 and Gli3 activators control ventral neuronal patterning by regulating progenitor segregation. Thus, multiple ventral neuronal types can develop in the absence of Gli function, but require balanced Gli protein activities for their correct patterning and differentiation.

Supplementary data available online

Key words: Sonic hedgehog, Gli genes, Patterning, Cell fate, Spinal cord, Sorting, Mouse, Chick

Introduction

In mammals, the secreted signaling protein Sonic hedgehog (Shh) is both necessary and sufficient to specify ventral cell fates in the developing central nervous system (CNS). The primary mediators of Shh signaling are the Gli zinc-finger-containing transcription factors. Three Gli genes have been identified in vertebrates – *Gli1*, *Gli2* and *Gli3*. These genes are homologous to the *Drosophila Cubitus interruptus* (*Ci*) gene, which is thought to mediate most, if not all, of the known responses to Hedgehog (Hh) signaling in this organism (Methot and Basler, 2001). It is not clear whether an obligatory role for *Gli/Ci* genes in mediating Hh signaling has been conserved from flies to vertebrates.

The *Ci* gene encodes a zinc-finger-containing transcription factor with two distinct activities, repression and activation (Aza-Blanc and Kornberg, 1999). Hh signaling controls these dual activities by promoting the formation of a full-length Ci activator protein at the expense of the shorter repressor form that is constitutively generated by partial proteolysis in the absence of Hh (Aza-Blanc et al., 1997). The differential sensitivity of Hh target genes to the two forms of Ci indicates that activation of Hh target genes involves both disinhibition and direct activation (Muller and Basler, 2000). Similarly, evidence suggests that vertebrate Gli2 and Gli3, but not Gli1, proteins can be cleaved to generate repressor forms, analogous to Ci (Dai et al., 1999; Wang et al., 2000; Aza-Blanc et al., 2000). However, the dual transcriptional activities embodied in Ci appear to be unequally distributed among the three vertebrate Gli proteins. Both gain- and loss-of-function studies indicate that Gli1 and Gli2 are the primary Gli activators that function downstream of Shh signaling, but only Gli2 is required in developing mice (Ruiz i Altaba, 1999; Ding et al., 1998; Matise et al., 1998; Bai et al., 2002). Gli3, however, appears to function as the primary Gli repressor (Wang et al., 2000), although biochemical and genetic evidence suggests that Gli3 can also function as an activator under certain circumstances (Dai et al., 1999; Shin et al., 1999; Motoyama et al., 2003; Bai et al., 2004). A repressor role for Gli2 has not been demonstrated.

All three Gli genes are expressed in spinal cord progenitor cells during early neurogenesis when cell fate specification is occurring (Sasaki et al., 1997; Platt et al., 1997; Lee et al., 1997). However, mouse mutant studies reveal only a limited role for each individual Gli gene in dorsoventral (DV) patterning in the spinal cord. Targeted *Gli2* mutant mice lack floor plate (FP) and most V3 cells that develop near the ventral midline, but other ventral cell classes are present in their normal DV positions, except motoneurons (MNs), which extend across the midline (Ding et al., 1998; Matise et al., 1998). *Gli1* mutants have no discernable spinal cord phenotype, even on a *Gli2* mutant background (Matise et al., 1998; Park et al., 2000), while loss of *Gli3* has only a subtle effect on the position of interneurons that develop in the intermediate region of the spinal cord (Persson et al., 2002).

By contrast, *Shh* mutants have a severe phenotype including cyclopia and an absence of most ventral cell types along the entire neuraxis (Chiang et al., 1996). In *Shh;Gli3* and *Smo;Gli3* double mutants, many ventral cells are rescued except FP and V3 interneurons (Litingtung and Chiang, 2000; Wijgerde et al., 2002), indicating that an important function of Hh signaling is to oppose the repressive activities of Gli3 in the ventral spinal cord. In addition, these results, and others (Krishnan et al., 1997), suggest the possibility that a Hh/Gli-independent pathway could mediate some aspects of Shh signaling in the ventral spinal cord. However, as Gli2 is still expressed in *Shh;Gli3* and *Smo;Gli3* mutants, a Hh-independent role for this factor in generating some ventral cell types cannot be ruled out.

Together, these studies suggest a model whereby graded Shh signaling controls the balance between Gli activator and repressor activities in progenitor cells along the DV axis, and predicts that the summation of Gli activities at specific Shh concentrations (and DV levels) will control distinct cell fates, ultimately by regulating expression of progenitor fate determinants (Stone and Rosenthal, 2000; Jacob and Briscoe, 2003). However, a number of issues remain unresolved. First, it is unclear which specific cell fates and progenitor determinants are controlled by the different Gli activities. For example, while it has recently been demonstrated that many ventral class II determinants are sensitive to Gli3 repression (Persson et al., 2002; Meyer and Roelink, 2003), whether and how specific Gli activator activities are involved in controlling the expression of these factors in spinal cord DV patterning has not been resolved. Second, it is unclear whether Gli3 plays a positive role in mediating Hh signaling. Indeed, it has recently been shown that loss of *Gli2* or *Gli3*, but not *Gli1*, can reverse aspects of the *Ptch1*$^{-/-}$ mutant phenotype in which the Hh pathway is constitutively activated (Bai et al., 2002; Motoyama et al., 2003), suggesting that they both possess the ability to transduce Hh signaling as activators in vivo. Finally, it is uncertain whether the Hh-dependent establishment of neuronal patterning and ventral cell fates requires all Gli protein functions. Resolving this issue is the rate-limiting step in determining the contribution of Hh-independent pathways to ventral cell fate specification and patterning.

In this study, we have addressed these issues using two approaches. First, we generated mouse embryos lacking both *Gli2* and *Gli3* genes and analyzed spinal cord development during early neurogenesis. As *Gli1* is not expressed in double homozygotes, all Gli protein activities are absent in these mice, providing an opportunity to address the requirement for all three vertebrate Gli factors and their combined activities in mediating Hh signaling in the developing spinal cord. To complement these studies, we employed gain-of-function experiments in chick embryos to study the individual transcriptional activities of Gli2 and Gli3. Our results show that motoneurons and three ventral interneuron subclasses are generated in *Gli2;Gli3* mutants, except floorplate and V3 cells, but strikingly these cells develop as intermingled populations. Furthermore, we show that Gli3 contributes activator functions to ventral neuronal patterning, playing a redundant role with Gli2. The similarities of these results to previously published studies (Litingtung and Chiang, 2000; Wijgerde et al., 2002), indicate that Gli proteins mediate all of the patterning functions of Hh in the developing spinal cord. We also show that activation of the Shh pathway in chick dorsal neural tube cells using Gli2 and Gli3 activator constructs, as well as expression of Shh, elicits cell clustering. Together, these results indicate both distinct and partially overlapping roles for Gli2 and Gli3 activator in patterning and cell fate specification in the ventral spinal cord downstream of Shh signaling, and suggest an important role for these activities in establishing or maintaining the segregation of ventral progenitors in discrete pools.

Materials and methods

Generation of *Gli2*$^{-/-}$*;Gli3*$^{xt/xt}$ double mutant mouse embryos

Gli2$^{-/-}$*;Gli3*$^{xt/xt}$ double-mutant embryos were generated by crossing mice heterozygous for a targeted mutation in *Gli2* (Mo et al., 1997) with the *extra-toes* (*Xt*^{J}) mutant mice containing an intragenic deletion in the *Gli3* gene (Hui and Joyner, 1993). All lines were maintained on a Swiss Webster background. Double-homozygous embryos, identified using PCR as previously described (Mo et al., 1997), were collected for analysis at embryonic day (E) 9.5 (n=7), E10-10.5 (n=11) and E11.5 (n=2).

In ovo electroporation in chicken embryos

cDNAs encoding amino acids 280-1544 of mouse Gli2 and 345-1596 of mouse Gli3 were cloned into the bi-cistronic expression vector pCIG (Sasaki et al., 1999; Megason and McMahon, 2002). An ATG codon was added in frame upstream of both constructs, and was confirmed by sequencing. For co-electroporation of these constructs with ptcΔloop2, the GFP was removed and ptcΔloop2 cDNA (Briscoe et al., 2001) was subcloned into pCIG. Five 25 V pulses were delivered for 50 mseconds at 1 second intervals at HH stages 12-14. Embryos were sacrificed 24 or 48 hours later and processed for analysis. At least 10 embryos were analyzed for each set of experiments. Noon on the day when vaginal plugs were detected was designated E0.5.

Immunohistochemistry, RNA in-situ hybridization and RT-PCR

Embryo collection, antibody staining and RNA in situ hybridization was performed as described (Matise et al., 1998). Antibodies used were mouse anti-BrdU (Sigma), cyclin D1 (Upstate Biotechnology), Shh, Nkx2.2, Hb9/Mnr2, Isl1, Pax6, Pax7, Foxa2 (DSHB), Gata3, Jag1 (Santa Cruz), Mash1 and Ngn1 (D. Anderson); rabbit anti-phosphorylated caspase 3 (Idun Pharmaceuticals), Dbx1, Dbx2, Nkx6.1 (T. Jessell), Chx10 (K. Sharma) and Olig2 (H. Takebayashi); and guinea-pig anti-Nkx2.9, Nkx6.2 (J. Ericson) and Evx1 (T. Jessell). Fluorochrome-conjugated secondary antibodies were obtained from Jackson ImmunoResearch or Molecular Probes. RNA in situ probes were mouse *Ptch1* (M. Scott), chicken *Ptch1* and *Ptch2* (C. Tabin). RT-PCR was performed as described using primers specific for mouse *Gli1* (Park et al., 2000).

Quantification of neurons and precursors was performed by averaging counts from wild-type and double-homozygous embryos. At least four sections for each set of markers were counted in three to eight different embryos at similar anterior-posterior levels.

Results

Expression of Shh target genes in *Gli2*$^{-/-}$*;Gli3*$^{xt/xt}$ mutant embryos

During early neurogenesis, the expression of *Gli2* and *Gli3* overlaps extensively in the spinal cord ventricular zone (VZ), although by E10.5, after neural tube closure, *Gli3* becomes repressed in the ventral most region with only weak levels persisting in motoneuron progenitors (Sasaki et al., 1997; Platt et al., 1997; Lee et al., 1997) (Fig. 1A,B). To determine the

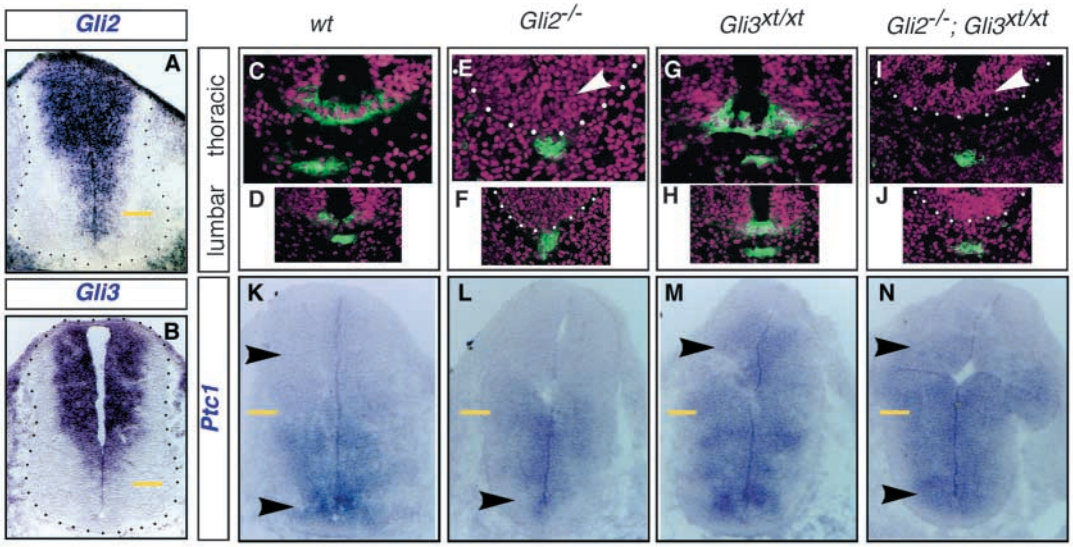

Fig. 1. Defects in Shh signaling in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutant embryos. (A,B) Widespread and overlapping *Gli2* and *Gli3* expression in the ventral spinal cord at E10.5. *Gli2* expression extends further ventral than *Gli3* at this stage (yellow bars mark similar DV position). (C-J) Shh protein expression in the notochord and floorplate (FP). (C,D) Wild-type embryos express Shh in the notochord and FP. (E,F) In *Gli2*$^{-/-}$ mutants, FP expression of Shh is selectively lost (arrowhead), while notochord expression is retained. (G,H) In *Gli3*$^{xt/xt}$ mutants, Shh expression in the notochord and FP is similar to that in wild type. (I,J) *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants do not show Shh expression in FP (arrowhead) but notochord expression is similar to *Gli2*$^{-/-}$ mutants. (K-N) *Ptch1* mRNA expression. Yellow lines indicate wild-type dorsal expression boundary. (K) In wild-type embryos, *Ptch1* expression is restricted to the ventral VZ, with higher levels in cells dorsal to the FP (lower arrowhead). *Ptch1* is absent in the dorsal VZ (upper arrowhead). (L) In *Gli2*$^{-/-}$ mutants, strong ventral *Ptch1* expression is not detected (arrowhead), but remaining VZ expression is similar to wild type. (M) In *Gli3*$^{xt/xt}$ mutants, *Ptch1* expression in the ventral VZ is similar to wild type, but ectopic expression is detected in the dorsal VZ (arrowhead). (N) In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, weak uniform *Ptch1* expression is seen (*n*=3) even in dorsal regions (upper arrowhead). All sections are from E10.5 embryos.

redundant functions of *Gli2* and *Gli3* in the spinal cord, we generated mice lacking both of these factors by crossing existing targeted *Gli2*$^{zfd/+}$ mice (Mo et al., 1997) with naturally occurring *extra toes*^{J} (*Xt*^{J}) mice that contain an intragenic deletion in *Gli3* (*Gli3*^{xt}) (Hui and Joyner, 1993; Buscher et al., 1998). Shh target gene expression and neural patterning was analyzed in spinal cords of *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ double-homozygotes during early neurogenesis at E9.5-E11.5.

We first examined *Gli1* expression as its transcription is under the direct control of Shh (Bai et al., 2002). In *Gli2*$^{-/-}$ mutants, *Gli1* expression is greatly reduced (Ding et al., 1998; Matise et al., 1998), indicating that its expression in the spinal cord depends primarily on *Gli2*. Notably, no *Gli1* expression was seen in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants using both RNA in situ hybridization and RT-PCR (data not shown). Thus, both Gli3 and Gli2 are necessary for Shh-dependent activation of *Gli1*. In addition, these results show that expression of all three mouse Gli genes is absent in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants.

In wild-type embryos, Shh expression is first seen in notochord precursors and then later in prospective floorplate (FP) cells in the midline of the neural plate. At E10.5, after neural tube closure, Shh expression is seen in both the FP and notochord (Fig. 1C,D). In *Gli2*$^{-/-}$ mutant embryos that lack a FP, Shh is detected only in the notochord (Fig. 1E,F) (Ding et al., 1998; Matise et al., 1998), while *Gli3*$^{xt/xt}$ mutants are indistinguishable from wild type (Fig. 1G,H). In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, Shh was detected in the notochord at all axial levels examined, but as in *Gli2* mutants the FP did not form and no Shh expression was seen in the ventral midline (Fig. 1I,J).

Ptch1 expression is a reliable indicator of Shh signaling as its transcription is de-repressed by Shh (Goodrich and Scott, 1998). In wild-type embryos, *Ptch1* expression is strong in VZ cells near the ventral midline and weaker in the ventral VZ, but is undetectable in the dorsal VZ (Fig. 1K). In *Gli2*$^{-/-}$ mutants, weak *Ptch1* expression was seen in the ventral VZ but the strong expression adjacent to the FP was not detected, probably owing to the absence of most V3 cells in these embryos (Fig. 1L). By contrast, *Gli3*$^{xt/xt}$ mutants showed strong ventral expression adjacent to the FP as in wild type, but weak ectopic expression was also detected in the dorsal VZ (Fig. 1M), suggesting that *Gli3* normally represses *Ptch1* transcription here. Interestingly, in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ double mutants, low levels of uniform *Ptch1* expression were detected in a pattern that is essentially a composite of the *Gli2* and *Gli3* single mutant patterns (Fig. 1N). By contrast, *Ptch2*, which is expressed only at low levels in the neural tube (Motoyama et al., 1998), was not affected in double mutants (data not shown). These results show that *Ptch1* can be transcribed at basal levels in the absence of Gli activity but requires both Gli2 and Gli3 to establish its normal pattern of expression in the spinal cord.

We noted overgrowth of the neuroepithelium in the thoracic regions of some *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, suggesting a proliferative defect (*n*=3/11 embryos at E10.5; see Fig. S1 at http://dev.biologists.org/supplemental; see also Fig. 1N, Fig. 3R) This was accompanied by an increase in cell cycle markers in affected areas, but was not confined specifically to dorsal or ventral regions (see Fig. S1 at http://dev.biologists.org/supplemental). The majority of *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants did not exhibit this phenotype, and cell proliferation was similar to wild type (see Fig. S1 at http://dev.biologists.org/supplemental). This finding indicates that loss of both *Gli2* and *Gli3* lead to sporadic defects in cell proliferation independent of patterning defects, which were confined to the ventral spinal cord.

Absence of V3 interneurons in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutant embryos

V3 interneurons that express Sim1 develop adjacent to the FP and derive from progenitors expressing Nkx2.2 and Nkx2.9

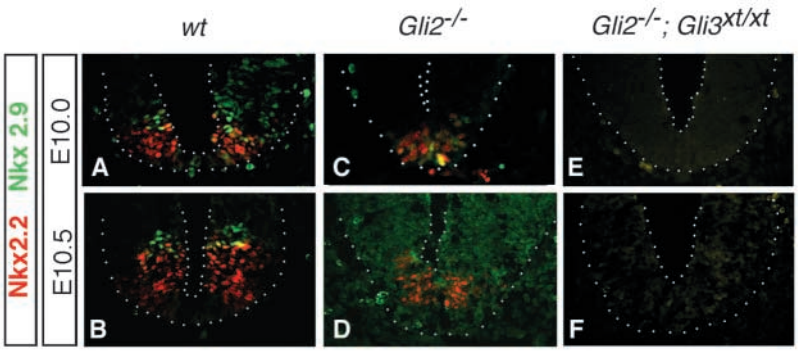

Fig. 2. Complete absence of V3 interneurons in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants. (A,B) In wild-type embryos, Nkx2.2 and Nkx2.9 mark V3 ventral interneuron progenitors that form adjacent to the FP. (C,D) In *Gli2*$^{-/-}$ mutants, Nkx2.2 expression is greatly reduced, and only a few Nkx2.9 cells could be detected at E10 (C) but not at E10.5 (D). (E,F) In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, neither protein was detected at any stages or axial level examined.

proteins (Fig. 2A,B) (Ericson et al., 1997; Briscoe et al., 1999). These cells are greatly reduced in number in the thoracic and lumbar spinal cord of *Gli2*$^{-/-}$ mutant embryos (Fig. 2C,D) (Ding et al., 1998; Matise et al., 1998), while *Gli3*$^{xt/xt}$ mutants are indistinguishable from wild type (data not shown). In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ double mutants, Nkx2.2, Nkx2.9 and Sim1 were not detected at any stage in either region (Fig. 2E,F). Thus, in the absence of both Gli2 and Gli3, V3 interneurons are not specified.

Alterations in motoneuron and ventral interneuron patterning and number in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ embryos

Our previous analysis of *Gli2*$^{-/-}$ mutant embryos showed that MNs are present in similar numbers as in wild type, but extend across the ventral midline (Ding et al., 1998; Matise et al., 1998). As both *Gli2* and *Gli3* are expressed in MN progenitors in the mouse spinal cord (Fig. 1A,B), it is possible that they are both required for the development of this cell type. Indeed, a recent study reported absence of MNs in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutant embryos at E9.5 (Motoyama et al., 2003). However, our analysis of later stage (E10.0-11.5) double mutant embryos revealed that MNs are present in both thoracic and lumbar regions, but in significantly reduced numbers, compared with wild type (Fig. 3A-C). This reduction was not due to increased MN apoptosis in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants (see Fig. S1 at http://dev.biologists.org/supplemental).

Three interneuron subclasses develop in the ventral spinal cord dorsal to MNs and can be identified by their expression of Chx10 (V2), En1 (V1) and Evx1 (V0). V2 cells can be further subdivided into two subclasses, V2a (Chx10+) and V2b (Gata3+), that develop as intermingled cells (Karunaratne et al., 2002) (Fig. 3D). In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, V1, V2a and V2b cells were present but were not confined to their normal domains dorsal to MNs (Fig. 3B,E,H). Rather, these cells expanded into the ventral midline where they were intermingled with one another and MNs in both thoracic (Fig. 3H,K) and lumbar (data not shown) regions. V0 neurons, by contrast, remained largely in their normal domain, although some mixing with V1 cells was seen at their dorsal boundary (Fig. 3H). Cell counts revealed a significant increase in V0 and V1 cells in thoracic, but not lumbar, regions at both E10.5 (Fig. 3I) and 11.5 (data not shown). By contrast, fewer V2 cells were found in both regions, similar to MNs (Fig. 3F). In no case did we observe inappropriate co-expression of neuronal markers in double mutant embryos at E10-10.5. Thus, in the absence of Gli activity, MN, V2 and V1 interneurons are generated as intermingled, rather than distinct, cell populations.

Abnormal MN differentiation and intermingling of MN, V1 and V2 progenitors in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants

To study the reduction in MN numbers in double mutant embryos, we examined the expression of MN progenitor (pMN) factors. In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, Olig2+ pMN cells were shifted ventrally in both thoracic and lumbar regions (Fig. 3L-P). Interestingly, in thoracic regions a higher numbers of pMN cells were seen in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants compared with wild type (~20% increase), while in lumbar regions there were fewer (~15% decrease) (Fig. 3N; n=21 sections scored in three embryos; data not shown). Thus, the reduction in postmitotic MNs generated in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants is not due solely to depletion of pMN cells. In addition, this decrease was not correlated with upregulation of cyclin D1 or downregulation of Ngn1 expression in pMN cells, nor increased MN apoptosis in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants (see Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants in both thoracic and lumbar regions could be due to a delay or decrease in the progression of motoneuron progenitors to post-mitotic neurons.

We next examined Pax6, which shows weak expression in pMN cells and strong levels in p2, p1 and p0 progenitor cells (Fig. 3Q) (Ericson et al., 1997). In *Gli2* mutants, Pax6 is repressed in the ventral pMN domain, a finding that could be explained by reduced but persistent Nkx2.2/Nkx2.9 expression in these mice (Fig. 2C,D) (Ding et al., 1998; Matise et al., 1998). Consistent with this, in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutant embryos, both Nkx2.2 and Nkx2.9 are completely absent and Pax6 expression extended into the ventral midline at E10.5 and E11.5 (Fig. 3R; data not shown). By contrast, Pax7 expression, which marks dorsal progenitors, was not altered in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants (data not shown).

In the ventral spinal cord, the five cardinal neuronal subtypes derive from progenitor domains (ventrally to dorsally: p3, pMN, p2, p1 and p0) in which cells express unique HD and basic helix-loop-helix (bHLH) proteins (Briscoe and Ericson, 2001). pMN and p2 cells can be identified by their expression of Olig2 and Mash1, respectively, while both progenitor domains express Nkx6.1 (Fig. 4A) (Lu et al., 2002; Zhou and Anderson, 2002; Parras et al., 2002). In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, Nkx6.1+/Mash1+ p2 progenitors were scattered throughout the ventral spinal cord (Fig. 4B). Dbx2, which normally defines the p1, p0 and pd6 progenitor domains (Pierani et al., 1999), was also detected in scattered cells throughout the ventral spinal cord in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants (Fig. 4C,D). By contrast, Dbx1 expression, which marks

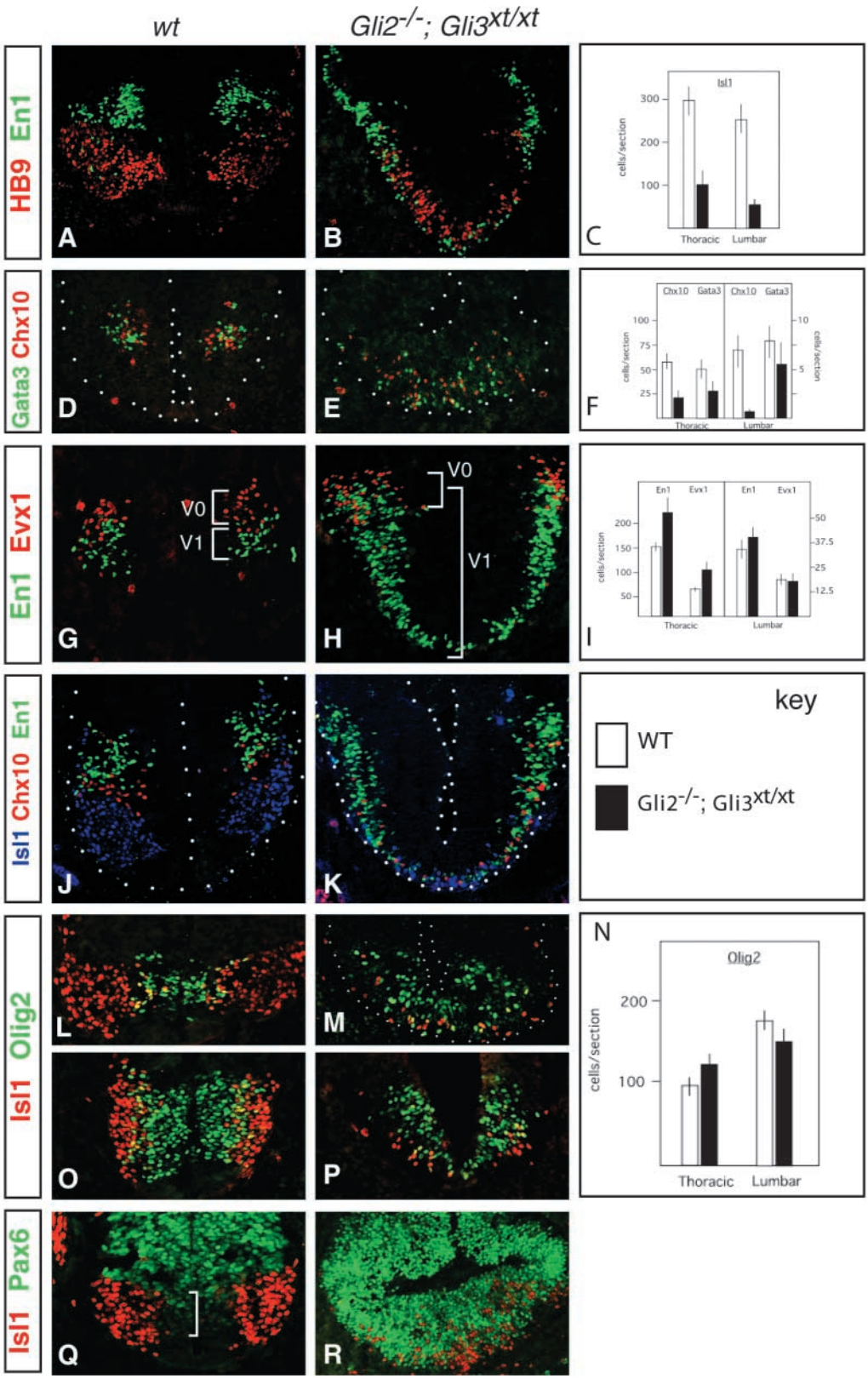

Fig. 3. MNs, V2 and V1 cells are generated in the ventral spinal cord of *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutant embryos but are intermingled. (A-C) MNs (HB9+) and V1 (En1+) cells are generated in double mutants but extend into the ventral midline. (C) Quantification of MN number in thoracic and lumbar regions of wild-type and *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants. (D,E) In wild-type embryos, V2a (Chx10) and V2b (Gata3) cells develop as intermingled sub-populations dorsal to V1 cells. In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, both classes of V2 cells were generated in the ventral midline but retained their intermingled organization. (F) Quantification of V2a and V2b cells in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, compared with wild type. (G,H) In wild-type embryos, V0 (Evx1+) and V1 (En1+) cells differentiate as discrete populations. In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, V0 cells show a limited expansion into the V1 domain. V0 and V1 intermingling is less pronounced at lumbar levels (data not shown). (I) Quantification of V1 cells in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants. (J,K) MNs, V2 and V1 neurons intermingle in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants. (L-P) Altered number of Olig2+ pMN cells in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants. Sections through mid-thoracic (L,M) and lumbar (O,P) regions. (N) Quantification Olig2 pMN numbers in wild-type and *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants. Histograms indicate average cell counts/section, error bars indicate s.e.m. (Q,R) Pax6 expression in pMN cells. Pax6 is normally expressed weakly in dorsally derived MNs (bracket, Q). In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, strong Pax6 expression extends to the ventral midline (R).

p0 cells (Pierani et al., 1999), was confined to its normal intermediate domain, consistent with the localization of V0 interneurons to this region in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants (data not shown).

As Dbx2 is expressed in multiple progenitor domains, we examined Jag1 expression to further define the identity of ectopic Dbx2 cells in the ventral spinal cord of *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants. Jag1 is expressed specifically in the p1 and pd6

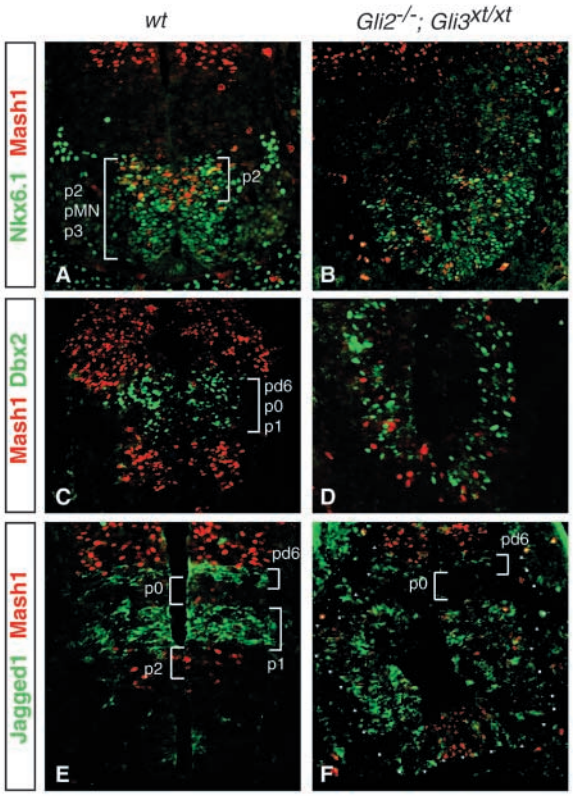

Fig. 4. p1 and p2 progenitors expand into the ventral spinal cord and intermingle in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants. (A,B) In wild-type embryos, Mash1 is co-expressed with Nkx6.1 in p2 progenitors. In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, Mash1+/Nkx6.1+ p2 progenitors are scattered throughout the Nkx6.1 domain. (C,D) In wild-type embryos, p1, p0 and pd6 progenitors express Dbx2 and are distinct from Mash1+ p2 progenitors ventrally and dorsally. In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, Dbx2+ and Mash1+ progenitors are intermingled in the ventral spinal cord, but co-expression of these two factors was not seen. (E,F) In wild-type embryos, Jag1 is expressed between Mash1 domains, but is excluded from the p0 domain. In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, only the ventral Jag1 expression domain in p1 progenitors has expanded ventrally, while the more dorsal domain, as well as the intervening Jag1-negative domain, were similar to wild type. Co-expression of Jag1 and Mash1 was not detected.

domains but is excluded from the p0 domain (Fig. 4E) (Matise and Joyner, 1997). In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, only the ventral domain of Jag1 that marks the p1 domain was expanded ventrally (Fig. 4F). No cells were seen that co-expressed Mash1 and Jag1. Thus, p1 and p2 progenitors remain segregated, even though they both expand into the ventral spinal cord in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants.

Olig2 expression in pMN cells is flanked by Mash1 expression in p3 and p2 cells (Fig. 5A). In *Gli2*$^{-/-}$ mutants, the ventral domain of Mash1 is absent, but the pMN and p2 domains segregate normally (Fig. 5B). By contrast, in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ double mutants, Mash1+ (p2) and Olig2+ (pMN) cells were intermingled throughout the ventral region, but no cells were found that co-express these factors (Fig. 5C). Thus, pMN and p2 progenitors are intermingled but distinct in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants.

In wild-type embryos at E10.5, Nkx6.2 expression in the VZ marks p1 and a small number of p0 progenitors, as well as a population of postmitotic MNs (Vallstedt et al., 2001) (Fig. 5D). In *Gli2*$^{-/-}$ mutants, expression of Nkx6.2 in the p1 and p0 domains was not altered, but expression persisted in postmitotic MNs, which extended across the midline (Fig. 5E).

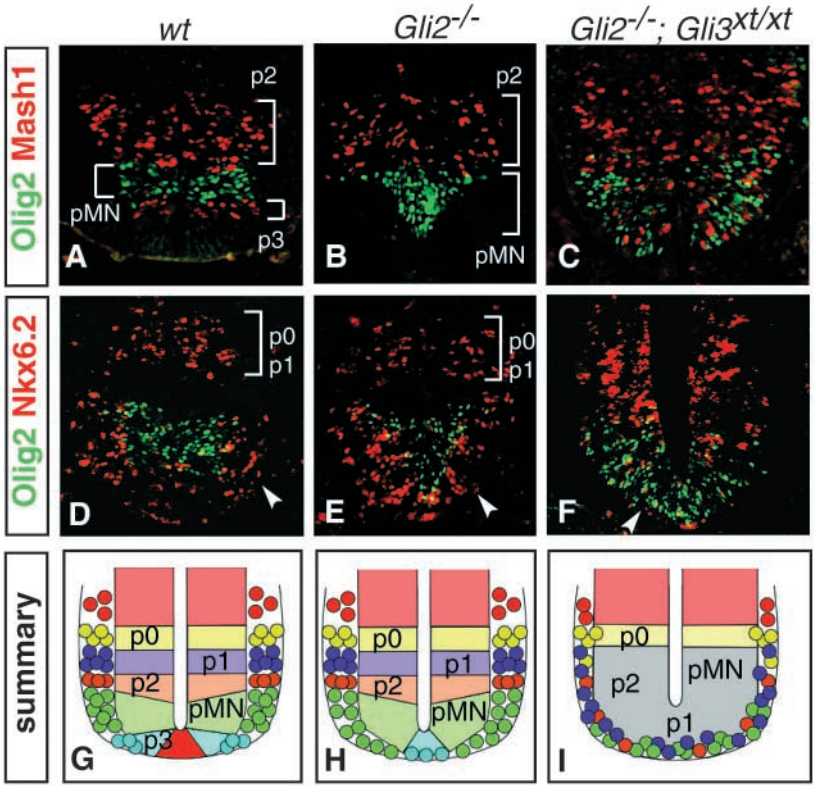

Fig. 5. pMN progenitors intermingle with p2 and p0 progenitor in the ventral VZ in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants. (A) In wild-type embryos, Olig2 and Mash1 are expressed in non-overlapping domains in the ventral spinal cord. (B) In *Gli2*$^{-/-}$ mutants, the Olig2 pMN domain expands into the ventral midline, but normal segregation between p2 Mash1+ and Olig2+ pMN cells is maintained. (C) In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, Olig2- and Mash1-expressing cells are intermingled, but no cells co-expressing these factors were found. (D) In wild-type embryos, Nkx6.2 marks the p1 and p2 progenitor domains in the VZ, as well as some post-mitotic MNs that form outside the Olig2+ pMN domain (arrowhead). (E) In *Gli2*$^{-/-}$ mutants, the dorsal Nkx6.2 domain in p0 and p1 progenitors is not altered, but ventrally Nkx6.2+ MNs extend across the midline (arrowhead), matching the ventral shift in Olig2+ pMN cells. (F) In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, VZ cells expressing Nkx6.2 expanded into the ventral midline, and these cells are intermingled with Olig2+ pMN cells. No cells were found that co-express these factors. Nkx6.2 expression was also detected in some post-mitotic MNs that were located outside of the Olig2+ expression VZ domain in *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants (arrowhead). (G-I) Schematics summarizing progenitor patterning in wild type, *Gli2*$^{-/-}$ and *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants.

In *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants, VZ cells expressing Nkx6.2 extended into the ventral spinal cord, but no cells co-expressing Olig2 and Nkx6.2 were detected (Fig. 5F), showing that p1 and pMN progenitors are intermingled but separate in these embryos.

These data show that in the absence of Gli activities, pMN, p2 and p1 progenitors are generated in the ventral spinal cord and maintain their distinct identities, despite developing as intermingled populations (summarized in Fig. 5G-I).

Dominant Gli2 and Gli3 activator proteins induce ventral and repress dorsal cell fates

Our analysis of *Gli2*$^{-/-}$;*Gli3*$^{xt/xt}$ mutants shows that Gli2 and Gli3 play redundant roles in the ventral spinal cord. However, loss-of-function studies do not permit us to address which specific Gli activity – activation or repression – is involved, because all are absent in double mutants.

To investigate this, we assayed the expression of ventral HD and bHLH fate determinants in the spinal cord of chick embryos after transfection, shortly after neural tube closure, of dominant constitutive Gli2 and Gli3 activator constructs. To generate these, we deleted the region encoding the N-terminal repressor domains of Gli2 and Gli3 (Sasaki et al., 1999; Pearse et al., 1999; Murone et al., 2000; Dunaeva et al., 2003) to generate Gli2ΔN-term and Gli3ΔN-term (see schematic, Fig. 6), and cloned these constructs into a bi-cistronic expression vector (Megason and McMahon, 2002) that also encoded GFP.

Transfection of either Gli2ΔN-term or Gli3ΔN-term shortly after neural tube closure did not induce ectopic Shh expression (Fig. 6A,A′,B,B′). This result is consistent with transplant studies that have defined a critical period for the induction of FP by notochord or ectopic Shh that ends prior to neural tube closure (Dodd et al., 1998).

We found that Gli2ΔN-term transfections induced cell-autonomous expression of multiple ventral markers, including Foxa2 (FP,V3), Nkx2.2 (V3), and (weakly) Nkx 6.1 (p3, pMN, p2) (Fig. 6C,E,G). By contrast, Gli3ΔN-term only induced Nkx 2.2 in these experiments (Fig. 6F). No effect was seen on Ngn2 or Nkx6.2 expression for either construct (data not shown). Both Gli2ΔN-term and Gli3ΔN-term were equally capable of repressing Pax6 and Pax 7 expression (Fig. 6I-L′). These results show that dominant Gli2 and Gli3 activator constructs

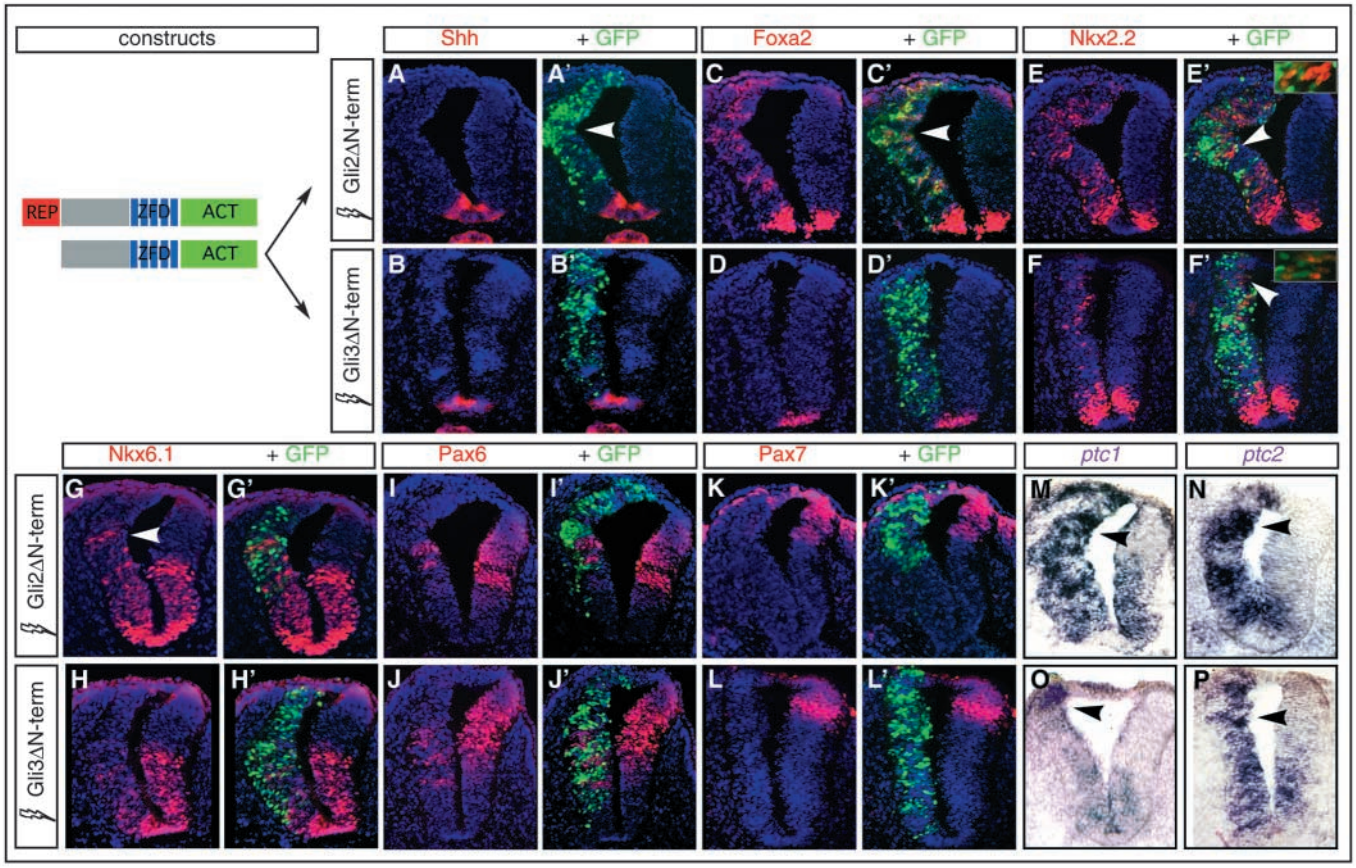

Fig. 6. Misexpression of dominant activator forms of Gli2 and Gli3 activates ventral and represses dorsal factors. (A-H′) Transfections of Gli2 and Gli3 cDNAs encoding N-terminally-truncated forms of the proteins (see schematic) using in-ovo electroporation in chick embryos. (A,B) Neither Gli2ΔN-term nor Gli3ΔN-term induced ectopic Shh expression when transfected at HH stage 12-14. Expansion of epithelium results from Gli2ΔN-term transfections (arrowhead; also see C′,E′). (C,D) Gli2ΔN-term induced weak expression of Foxa2/Hnf3β throughout the spinal cord, while Gli3ΔN-term did not. (E,F) Both Gli2ΔN-term and Gli3ΔN-term induced robust ectopic Nkx2.2 expression throughout the spinal cord. Insets show higher power view of area indicated by arrowhead, excluding the blue channel. (G,H) Gli2ΔN-term, but not Gli3ΔN-term, induced Nkx6.1 expression in the dorsal spinal cord. (I-L) Both Gli2ΔN-term and Gli3ΔN-term suppressed Pax6 and Pax7 expression in the dorsal spinal cord. (M,N) Gli2ΔN-term induced patchy upregulation of *Ptch1* and *Ptch2* transcription. (O,P) Gli3ΔN-term induced *Ptch1* weakly but *Ptch2* strongly.

are capable of inducing expression of a limited set of ventral Class II fate determinants, with Gli2ΔN-term showing a broader capability compared with Gli3ΔN-term, whose activity appears to be confined primarily to the induction of Nkx2.2.

Interestingly, ~1/3 of Gli2ΔN-term transfected embryos also exhibited an expansion of the neuroepithelium on the transfected side (*n*=7/20 embryos), which resulted in a unilateral increase in the expression of cell cycle markers (Fig. 6A′,C′,E′; data not shown). Gli3ΔN-term transfections, however, did not alter the size or shape of the neuroepithelium.

We next assayed whether Gli2ΔN-term and Gli3ΔN-term activator constructs could influence the transcription of *Ptch1* and *Ptch2*. Mis-expression of both factors upregulated strong *Ptch2* expression (Fig. 6N,P), while Gli2ΔN-term was more efficient than Gli3ΔN-term in inducing *Ptch1* (Fig. 6M,O). These results suggest that *Ptch1* and *Ptch2* are differentially responsive to Gli2 and Gli3 activators.

To rule out the possibility that Gli2ΔN-term or Gli3ΔN-term induced the expression of Shh at low levels that escaped detection, we co-transfected these constructs with ptc-Δloop2, a dominant inhibitor of Shh signaling (Briscoe et al., 2001). On its own, ptc-Δloop2 can block Nkx 2.2 and activate Pax7 expression (see Fig. S2 at http://dev.biologists.org/supplemental) (Briscoe et al., 2001). Co-transfection of Gli2ΔN-term or Gli3ΔN-term with ptc-Δloop2 overcame these effects and instead activated Nkx2.2 and repressed Pax7 expression, and for all markers examined co-transfection results were similar to Gli2ΔN-term or Gli3ΔN-term alone (see Fig. S2 at http://dev.biologists.org/supplemental; data not shown). These results show that alterations in gene expression after transfections with Gli2ΔN-term and Gli3ΔN-term activator constructs cannot be explained by the secondary induction of Shh, but instead are the result of these factors acting in a ligand-independent cell-autonomous manner to induce target gene expression.

Activation of the Shh pathway in dorsal cells elicits cell clustering

The non-uniform expression of induced *Ptch1* and *Ptch2* by Gli2 and Gli3 activators, and the expansion of the neuroepithelium elicited by Gli2 activator transfections indicate that these factors might possess activities that are not directly related to control of progenitor fate determinant gene expression. To study this, we examined the behavior of transfected cells within the Pax7 domain in the dorsal spinal cord. In these experiments, we were guided by similar studies in *Drosophila* wing imaginal discs which showed that manipulation of the Hh pathway in anterior and posterior compartment cells could modify cell affinities between Hh transducing and non-transducing cells (Dahmann and Basler, 2000). In addition, recent experiments in mice provided evidence for a similar function of the Hh pathway in spinal cord patterning. In this study, Hh signaling was blocked in ventral cells by generating chimeras with *Smo*$^{-/-}$ ES cells, and it was found that these cells ‘clumped’ together in the ventral spinal cord (Wijgerde et al., 2002). We reasoned that if the Shh pathway functioned in part to segregate ventral from dorsal cells, then activating the pathway in dorsal cells should also elicit similar clustering behaviors.

We first transfected Shh alone into the spinal cord and monitored Nkx2.2 and Pax7 expression to identify cells in which the pathway has or has not been activated, respectively. Not unexpectedly, ectopic Shh induced widespread, almost uniform expression of Nkx2.2, and repressed Pax7 in both cell-autonomous and non-autonomous manners (Fig. 7A,B,D). In this experiment, Nkx2.2-expressing cells did not segregate from one another or from dorsal cells. As a control for these experiments, we transfected GFP alone, which did not elicit cell clustering or alterations in either Nkx2.2 or Pax7 expression (Fig. 7C).

We next co-transfected Shh with ptc-Δloop2 to block autocrine Shh signaling in transfected cells but permit non-cell autonomous induction of Nkx2.2 expression (Fig. 7H). Efficient cell-autonomous blockage of autocrine Shh signaling was indicated by expression of Pax7 (indicating pathway is off) in GFP+ transfected cells (Fig. 7G). In these experiments, induced, untransfected cells expressing Nkx2.2 frequently segregated from transfected (but non-transducing) cells in the dorsal spinal cord (Fig. 7E,F; *n*=23 sections, 5/7 embryos). Significantly, clustering was not evident in ventral cells induced to express Nkx2.2 (7E,F′; *n*=8).

These results show that activation of the Shh pathway in dorsal cells can elicit cell clustering. To determine whether Gli activators mediated this effect, we transfected Gli2ΔN-term or Gli3ΔN-term constructs and examined transfected embryos that exhibited broad transfections extending into the dorsal spinal cord. In these experiments, we found that in a majority of sections examined (*n*=13/17 sections, 10 embryos), transfected cells that did not show Pax7 expression were found in clusters, surrounded by non-transfected Pax7+ cells (Fig. 7I-L), although ventrally located transfected cells were distributed more uniformly (*n*=14 sections; data not shown; Fig. 7M). Similar results were obtained at the lowest Gli2ΔN-term concentration that induced Nkx2.2 expression (*n*=3; data not shown). Thus, cell clustering elicited by activation of the Shh pathway in dorsal neural tube cells appears to be mediated by Gli2 and/or Gli3 activator activities (Fig. 7M,N).

To determine whether cell clustering that results from activation of the Shh pathway is mediated by induction of Nkx2.2, we transfected this factor alone and assayed the position of transfected cells in the dorsal spinal cord. In contrast to Shh or Gli2/Gli3 activator transfections, Nkx2.2-transfected cells did not segregate from non-transfected cells in the dorsal spinal cord in any case examined (*n*=8 sections, three embryos; data not shown). These results show that cell clustering induced by Shh-pathway activation is independent of Nkx2.2 induction in chick neural progenitors.

Discussion

In this report, we addressed the requirement for Gli protein activities in mediating Shh-dependent patterning and cell fate specification in the developing vertebrate spinal cord. Our analysis of mouse mutants lacking both *Gli2* and *Gli3* genes shows that these factors function redundantly in the specification of V3 interneuron and in DV patterning of multiple ventral neuronal classes. As *Gli1* is not transcribed in double homozygotes, all possible repressor or activator forms of Gli proteins are absent. We found that MN, V2, V1 and V0 cells were generated in altered numbers in *Gli2;Gli3* double mutants, and MN, V2 and V1 neurons and their precursors were expanded into the ventral midline but retained their

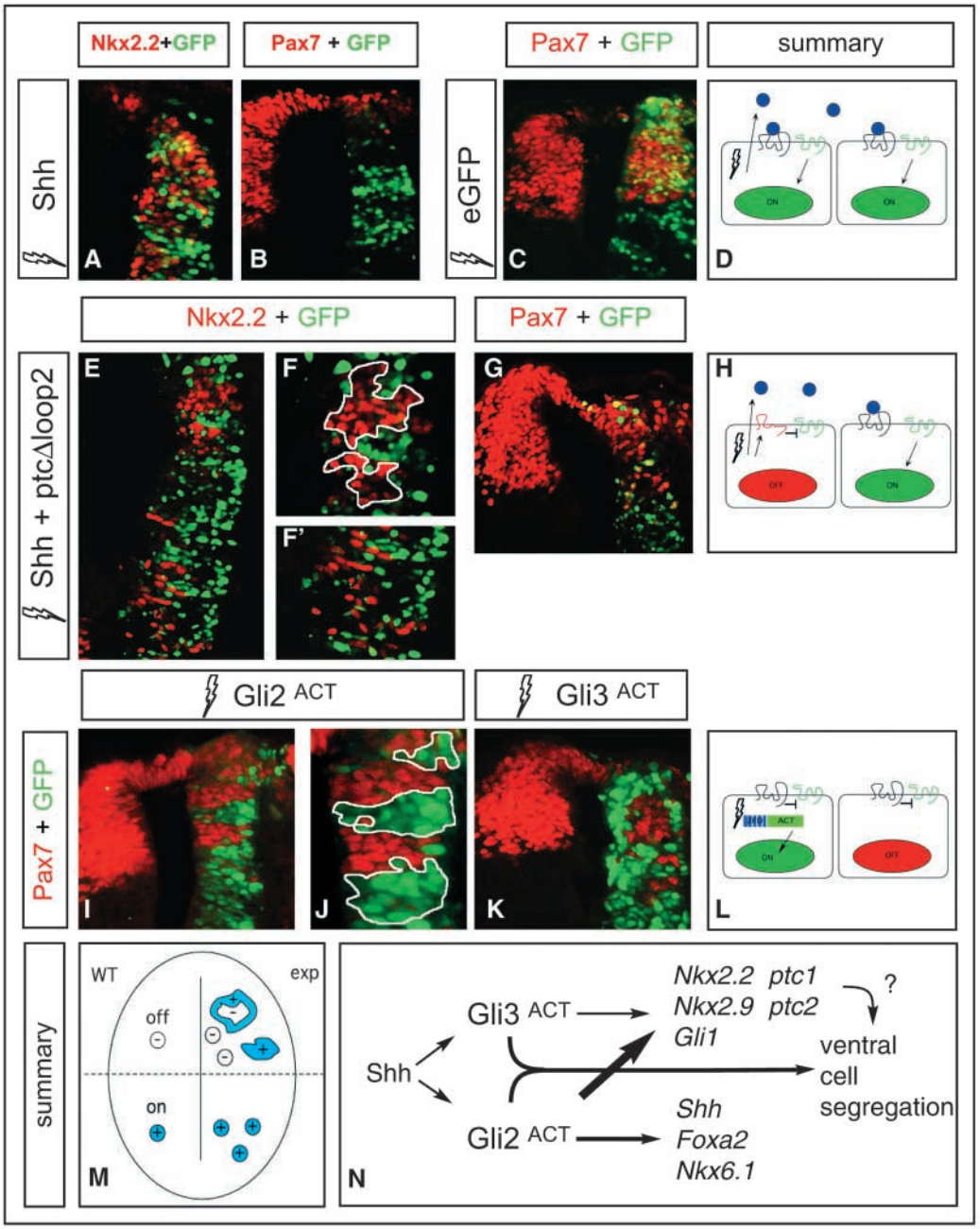

Fig. 7. Activation of the Shh pathway in dorsal progenitors in the chick neural tube elicits cell clustering. (A,B) Transfection of Shh (green) elicits widespread, relatively uniform activation of Nkx2.2 (red) and repression of Pax7 (red) cell autonomously and non-autonomously. (C) GFP-only transfections have no effect on endogenous Pax7 or Nkx2.2 (not shown) expression. (D) Schematic illustrating broad activation of the Shh pathway (indicated by Nkx2.2 expression) in transfected (via autocrine signaling) and untransfected (via paracrine signaling) cells. Transfected cell is indicated by a lightening bolt. (E,F) Co-transfection of Shh with ptcΔloop2 (green) induces Nkx2.2 expression (red) only in untransfected cells. In this case Nkx2.2+ cells in the dorsal (F) but not ventral (F′) spinal cord segregate into clusters. (G) Expression of Pax7 (red) in co-transfected cells (yellow) indicates autocrine Shh signaling is blocked. (H) Schematic representation of signaling differences between untransfected and Shh + ptcΔloop2 co-transfected cells. Only paracrine Shh signaling occurs. (I-K) Cells transfected with Gli2ΔN-term or Gli3ΔN-term (green) activators form clusters in the dorsal spinal cord (J) that do not express Pax7 (red), while ventral cells do not cluster (not shown). (L) Schematic showing cell-autonomous, ligand-independent activation of the Shh pathway by transfections of dominant-activator Gli2 and Gli3 proteins. Untransfected cells retain their dorsal identity. (M,N) Schematic summarizing results of chick transfection studies and Shh/Gli pathway in ventral spinal cord progenitor cells. Shh pathway activation is indicated by blue '+' areas. Blue outlined areas in the dorsal region represent clusters of cells, while circles indicate individual cells. In M, arrow thickness indicates relative roles of Gli2 and Gli3 activators in inducing ventral target genes.

individual identities despite developing as intermingled populations. We show that Gli2 and Gli3 activator activities are sufficient and required to induce Nkx2.2 expression in V3 interneuron precursors. Furthermore, we show that N-terminally truncated forms of both Gli2 and Gli3 could activate a limited set of ventral genes, but that they differ in their ability to do so, suggesting that intrinsic functional differences between these proteins contributes to their unique roles in mediating Shh signaling. Finally, we show that activation of the Shh pathway in dorsal cells elicits their segregation from non-transducing dorsal cells. Together, these results raise the possibility that Shh/Gli signaling functions in neuronal patterning by controlling progenitor cell segregation.

Unique and redundant roles for Gli2 and Gli3 activators in patterning ventral cells

Our current analysis of neuronal specification in *Gli2* single and *Gli2;Gli3* double mutants indicates that Gli3 is partially redundant with Gli2 for the expression of *Gli1* and the generation of V3 cells. In *Gli2* mutants, *Gli1* expression is

severely reduced and the FP and most V3 cells are absent (Matise et al., 1998), whereas in *Gli2;Gli3* mutants *Gli1* expression and V3 cells are completely absent. Our transfection studies, furthermore, show that dominant activator forms of both Gli2 and Gli3 are capable of inducing ectopic expression of the V3 marker Nkx2.2 throughout the neural tube. Together with previous studies (Briscoe et al., 1999; Aza-Blanc et al., 2000; Bai and Joyner, 2001; Bai et al., 2004), these results demonstrate that Gli2 and Gli3 activators function redundantly to define the p3 progenitor domain and specify V3 interneurons by controlling expression of *Nkx2*.2 and perhaps *Gli1*.

Both Gli2 and Gli3 contain an N-terminal repressor domain that physically interacts with Su(Fu), a negative regulator of the Shh pathway (Pearse et al., 1999; Murone et al., 2000; Dunaeva et al., 2003). Previous studies in transgenic mice showed that misexpression in the dorsal midbrain of Gli2 and Gli3 proteins lacking this N-terminal domain, but retaining the DNA-binding zinc finger and C-terminal activation domains (Yoon et al., 1998), could activate ectopic expression of Foxa2/Hnf3β (Sasaki et al., 1999). Interestingly, we found that misexpression of N-terminally-deleted Gli2 and Gli3 activators in chick spinal cords using in ovo electroporation elicited different outcomes. Gli2 activated expression of Foxa2 (Hnf3β), Nkx2.2 and Nkx6.1, as well as inducing an expansion of the neuroepithelium, while Gli3 was only capable of inducing Nkx2.2. The striking similarity of these activities to the differential requirements for Gli2 and Gli3 in specifying ventral cell fates, as revealed by our analysis of *Gli2;Gli3* double mutants, suggests that these might reflect functional differences in the capacity of Gli2 and Gli3 to activate Shh target genes. However, as N-terminally-truncated forms of Gli2 and Gli3 are not detected in situ, we cannot rule out the possibility that the different responses to Gli2ΔN-term and Gli3ΔN-term in our transfection experiments do not reflect true differences in the activities of these proteins in vivo. Nevertheless, these results suggest that intrinsic functional differences between Gli2 and Gli3 could play a role in differentiating the cellular responses to Shh.

We found that *Ptch1* and *Ptch2* are differentially responsive to constitutive Gli2 and Gli3 activators. Taken together with previous studies showing that a Gli3 repressor can downregulate *Ptch1* and *Ptch2* transcription in the neural tube (Persson et al., 2002), it is likely that *Ptch1* and *Ptch2* are regulated by both Gli activator and repressor activities in the developing spinal cord. In this regard, the Ptch genes appear analogous to *dpp* in flies, the expression of which is controlled by both activator and repressor forms of Ci (Muller and Basler, 2000).

Obligatory role for Gli proteins in mediating Hh-dependent neural patterning in the ventral spinal cord

Studies in *Drosophila* have shown all of the known responses to Hh signaling require the activities of Ci (Methot and Basler, 2001). In *Smo;Gli3* mutants, which are in theory incapable of responding to all Hh proteins, many ventral cell types that are lost in *Shh* mutants are rescued, but some populations are mixed in the ventral spinal cord (Wijgerde et al., 2002). However, as *Gli2* is still present in these mice as its transcription is independent of Shh signaling (Bai et al., 2002), it was not possible to conclude that all Gli protein activities are required for this potential function. Results from the present study reveal numerous phenotypic similarities between *Smo;Gli3* and *Gli2;Gli3* mutants, and provide support for the idea that Gli protein activities are required for all the known patterning functions of Hh signaling in the developing ventral spinal cord.

Role of Gli proteins in mediating graded Shh signaling in the developing spinal cord

From our studies and those from other laboratories, a clear picture has emerged of the individual roles of Gli protein activities in mediating cellular responses to graded Hh signaling in the developing spinal cord. All three Gli factors contribute positive functions to the transduction of the Shh signal in the ventral spinal cord, with Gli2 and Gli3 playing the predominant roles to transduce the initial Shh signal and Gli1, which is induced by Gli2 and Gli3, making a minor contribution. Thus, all three Gli proteins participate as activators in the induction of FP and V3 interneurons, while Gli2 and Gli3 activators are also required for normal motoneuron differentiation and V2-V0 interneuron patterning and number, but not specification. Gli3 serves as the primary Gli repressor that must be inhibited to allow normal ventral cell fate development, but this activity also appears to be required for normal V2-V0 interneuron development independent of Hh signaling (Persson et al., 2002).

Available evidence does not, however, definitively exclude the possibility that a Gli2 repressor could play a minor Hh-independent role in the spinal cord. The increase in V1 interneurons in thoracic regions of *Gli2;Gli3* double mutants compared with normal embryos could be due to the removal of a repressive function for Gli2 that normally serves to limit the number of these cells. Interestingly, the situation in *Smo;Gli3* embryos (that might retain Hh-independent Gli2 repressor activity) appears to be the converse of this, with slightly more V1 cells in lumbar, but not thoracic, regions (Wijgerde et al., 2002). These findings suggest that if Gli2 does function as a repressor in vivo, this activity is redundant with Gli3 and its role differs along the rostrocaudal axis of the spinal cord. However, no direct evidence has been provided to date demonstrating the presence of an active, truncated repressor form of Gli2 in the developing spinal cord. Alternatively, as it has been shown that V1 and V0 cells can be induced by retinoids (Pierani et al., 1999), it is also possible that the normal formation of these cell types involves a correct balance or integration of both Shh and retinoid signaling pathways, and that this balance is differently affected in mouse *Gli2;Gli3* and *Smo;Gli3* mutant backgrounds.

Our results also reveal a redundant role for Gli protein activities in normal motoneuron differentiation. We found that in mice lacking all Gli activities, postmitotic motoneurons were present in greatly reduced numbers relative to their precursors, which were either near normal in number or elevated (in thoracic regions). This defect is not explained by inappropriate up-regulation of cyclin D1 or downregulation of Ngn2 in motoneuron precursors (see Fig. S1 at http://dev.biologists.org/supplemental; Q.L and M.P.M., unpublished). One possibility is that in the absence of regulated Gli protein activities, motoneuron progenitors do not respond normally to differentiation cues that are transmitted to cells during or shortly after their terminal mitosis.

Gli2 and Gli3 activator activities contribute to Shh/Gli control of neuronal patterning by regulating progenitor segregation

Our analysis of neuronal progenitor markers in *Gli2;Gli3* mutant spinal cord reveals that p1 and p2 cells that develop in the ventral spinal cord are mixed with each other and pMN progenitors. We show that these progenitors maintain distinct identities despite being mislocalized in the ventral spinal cord. As a result, neural patterning is similarly disrupted, with V2, V1 and MNs forming as intermingled, rather than discrete, populations. Within the context of current models, the simplest interpretation of these results is that, in the absence of Gli activities, many ventral precursors are incapable of responding normally to graded Shh signaling, resulting in the random specification of progenitors and the generation of similarly intermingled neuronal progeny. However, another interesting explanation could also account for these results. Lineage tracing studies indicate extensive intermixing of spinal cord progenitors prior to neurogenesis (Erskine et al., 1998). It is therefore conceivable that the mechanisms involved in specifying positional identity in the spinal cord function in part by restricting this intermixing. This function has been proposed for HD progenitor fate determinants, the expression of which is initiated at the onset of neurogenesis and which function to refine domains by mutual cross-repression (Briscoe et al., 2000). However, with the exception of Nkx2.2, our results show that Gli-mediated Shh signaling is not required for the induction or cross-repressive functions of most ventral HD determinants but rather is needed to organize their normal expression into distinct DV domains.

Furthermore, our gain-of-function studies in chick embryos suggest a role for the Shh/Gli signaling in establishing progenitor pool segregation in the spinal cord that is independent of their regulation of HD determinants. In these experiments, we activated the Shh pathway in dorsal cells using overexpression of ligand or N-terminally truncated Gli2/Gli3 activator proteins. In both cases, cells in which the pathway was activated were segregated from non-transducing dorsal cells when assayed 24 hours after transfection. As cell clustering cannot be induced by Nkx2.2 (the only HD factor consistently induced in these assays), it appears that distinct Gli target genes mediate this effect.

A role for Hh signaling in controlling progenitor segregation has also been proposed based on recent studies in $Smo^{-/-}$ mouse mutants (Wijgerde et al., 2002). In this study, Hh signaling was blocked in ventral cells by generating chimeras with $Smo^{-/-}$ ES cells, and it was found that progenitors derived from these cells 'clumped' together in the ventral spinal cord. However, as *Smo*-mutant cells in chimeras are present throughout embryogenesis, it is unclear whether Smo (or Hh signaling) is required in these cells during neurogenesis (when cell fate specification is occurring) or at some earlier time point. In addition, any Gli proteins present within $Smo^{-/-}$ mutant cells are likely to be repressors, so whether Gli activators, or Gli proteins in general, also participate in the hypothesized role of Hh signaling in progenitor segregation was not addressed. Our results establish that the opposite manipulation (activating the pathway in dorsal cells) has a similar outcome. Together, these results demonstrate that altering Shh pathway status in cells relative to their neighbors, either positively (our study) or negatively (Wijgerde et al., 2002), can induce segregation. Furthermore, cell segregation defects are only seen in mouse mutants in which all Gli activator activities are absent ($Smo^{-/-}$ chimeras, *Smo;Gli3* and *Gli2;Gli3* mutants). Together, these results illustrate a crucial role for Gli activators, either on their own or in balance with Gli repressors, in controlling progenitor segregation in the neural tube.

It is not immediately obvious from our studies how Gli activator-induced cell clustering in the dorsal spinal cord is related to their normal role ventrally. Pathway-activated cells in the ventral spinal cord did not form clusters, even at 20-fold lower Gli2 activator concentrations or when co-transfected with Gli3 repressor (Q.L. and M.P.M., unpublished). This could be due to a requirement for co-factors [such as Fu or Su(Fu)] that are known to regulate Gli activity through binding to the N-terminal region deleted from our constructs (Murone et al., 2000), or to other modulators of Gli activator activity that function specifically in the ventral spinal cord.

Our studies do not address the mechanisms that are responsible for Shh/Gli-mediated progenitor segregation. In principle, regulation of either differential cell adhesion or proliferation rates, or some combination of the two, could be involved. In *Drosophila* wing imaginal discs, Hh signaling in anterior (A) cells controls their segregation from posterior (P) cells at the AP border (Tepass et al., 2002). Inactivation of Hh or Ci result in mutant cells exhibiting sorting behavior characteristic of P cells in which Hh signaling is normally blocked by En (Dahmann and Basler, 2000). Phenotypically similar results were obtained by manipulating cadherin levels, providing a potential link between Hh signaling and the control of differential cell affinity (Dahmann and Basler, 2000). The striking similarities in fly and vertebrate phenotypes that result from manipulating Hh/Ci/Gli signaling suggests that the mechanisms that control differential cell segregation downstream of Hh signaling are conserved through evolution.

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