Sonic Hedgehog Signaling Confers Ventral Telencephalic Progenitors with Distinct Cortical Interneuron Fates

Qing Xu,1 Lihua Guo,1 Holly Moore,2 Ronald R. Waclaw,3 Kenneth Campbell,3 and Stewart A. Anderson1,*

1Weill Cornell Medical College, 1300 York Avenue New York, NY 10065, USA
2Columbia University College of Physicians and Surgeons and New York State Psychiatric Institute, New York, NY 10032, USA
3Division of Developmental Biology, Cincinnati Children’s Hospital Medical Center, University of Cincinnati College of Medicine, 3333 Burnet Avenue, Cincinnati, OH 45229, USA
*Correspondence: saa2007@med.cornell.edu
DOI 10.1016/j.neuron.2010.01.004

SUMMARY

Interneurons in the cerebral cortex regulate cortical functions through the actions of distinct subgroups that express parvalbumin, somatostatin, or calretinin. The genesis of the first two subgroups requires the expression of NKX2.1, which is maintained by SHH signaling during neurogenesis. In this paper, we report that mosaic elimination in the medial ganglionic eminence (MGE) of Smo, a key effector of SHH signaling, reveals that MGE progenitors retain a remarkable degree of plasticity during the neurogenic period. SHH signaling prevents the upregulation of GSX2 and conversion of some MGE progenitors to a caudal ganglionic eminence-like, bipolar calretinin-expressing cell fate that is promoted by GSX2. In addition, a higher level of SHH signaling promotes the generation of the somatostatin-expressing interneuron at the expense of parvalbumin-expressing subgroup. These results indicate that cortical interneuron diversity, a major determinant of cortical function, is critically influenced by differential levels of SHH signaling within the ventral telencephalon.

INTRODUCTION

Cerebral cortical functions are mediated by the coordinated activity of glutamatergic projection neurons and GABAergic interneurons. The interneurons regulate cortical functions through the actions of subtypes that are distinguished by morphological, axon targeting, physiological, and neurochemical characteristics (Freund, 2003; Markram et al., 2004). Recent studies have demonstrated that distinct classifications of interneurons have distinct spatial and temporal origins. For example, the parvalbumin-expressing (PV+) and somatostatin-expressing (SST+) subgroups originate from the medial ganglionic eminence (MGE), whereas the major subgroup of calretinin-expressing (Cair+) interneurons, which has a vertically oriented, bipolar or bifurcated morphology, originates mainly from the caudal ganglionic eminence (CGE) (Butt et al., 2005; Wichterle et al., 2001; Xu et al., 2004). Within the MGE, there is a bias for SST+ interneurons to originate from the dorsal MGE, and the PV+ interneurons to originate from the ventral MGE (Flames et al., 2007; Wonders et al., 2008). In addition to spatial distinctions in interneuron origins, temporal distinctions in the birthdates of interneuron subgroups (Cavanagh and Pamarvelas, 1989; Rymar and Sadikot, 2007; Xu et al., 2004) or subtypes (Butt et al., 2005; Miyoshi et al., 2007) have also been identified.

Taken together, these studies provide strong evidence that some neurochemical and physiological aspects of interneuron fate are specified around the time of their birth in the ventral (sub-pallial or pallidal) telencephalon. However, despite their relevance to neuropsychiatric disorders, relatively little is known about the molecular regulation of cerebral cortical interneuron fates. Initial patterning of the MGE requires FGF and SHH signaling to induce or permit expression of Nkx2.1 (Chiang et al., 1996; Fuccillo et al., 2004; Gutin et al., 2006; Storm et al., 2006), a transcription factor that is required for normal MGE development (Sussel et al., 1999). During neurogenesis, continued SHH signaling is required to maintain Nkx2.1 expression in MGE progenitors (Anderson et al., 2001; Xu et al., 2004; Xu et al., 2005), and Nkx2.1 is required for the specification of SST+ and PV+ interneurons (Butt et al., 2008; Du et al., 2008; Xu et al., 2004). Lhx6, a direct target of Nkx2.1, is also required for the specification of SST+ and PV+ subgroups (Du et al., 2008; Liodis et al., 2007). This role of Nkx2.1 and Lhx6 in the specification of MGE-derived interneurons is perplexing with regard to the specification of SST+ versus PV+ subgroups, because neither of these transcription factors has an apparent gradient of protein expression on the dorsal-ventral axis of the MGE. However, the enriched expression of the SHH signaling effectors Gli1, Ptc1, Hhip1, and Nkx6.2 in the dorsal MGE suggests that SHH signaling is enhanced in this region relative to the ventral MGE (Wonders et al., 2008; Yu et al., 2009).

To test the hypothesis that higher levels of SHH signaling direct the SST versus PV interneuron subgroup fates, we eliminated the SHH signaling effector Smo (Smo) within progenitors of the MGE in Six3Cre;Smo mice. These mutants have a mosaic loss of SHH signaling within strips of cells in the MGE and a remarkable combination of cell-autonomous and cell-nonautonomous effects on progenitor patterning and fate that affect all three major subgroups of cortical interneurons. Downregulation of SHH signaling results in reduction of Nkx2.1 expression, upregulation of GSX2, and conversion of their fate in some instances from...
PV- or SST-expressing interneuron subgroups to bipolar Calr+ interneurons that normally originate primarily from the caudal ganglionic eminence (Butt et al., 2005; Xu et al., 2004). Gain and loss of function studies suggest that Gsx2 contributes to bipolar Calr+ interneuron specification. In addition, mosaic inactivation of SHH signaling in the MGE results in a cell-nonautonomous up-regulation of this signaling in neighboring progenitors, ectopic expression of Gli1, Ptc1 and Nkx6.2, and conversion of middle and ventral MGE progenitors to dorsal MGE-like, SST+ fate. Exposing ventral MGE progenitors to exogenous SHH in slice culture produces a similar effect on SST+ fate, while suppressing the generation of PV+ interneurons. These results indicate that interneuron fate is remarkably plastic during neuronogenesis, and that interneuron diversity is critically influenced by differential levels of SHH signaling within the pallidal telencephalon.

RESULTS

**Six3Cre Produces a Mosaic of Cre Expression within the Ventricular Zone of the MGE**

To produce a mosaic of Smo inactivation in the MGE, we used a mouse line in which the Cre recombinase is expressed under control of a roughly 9 kb genomic fragment flanking the first exon of the Six3 gene (Furuta et al., 2000). By embryonic day (E) 10.5, Six3Cre-mediated reporter expression is evident in the MGE region of the ventral telencephalon (Figure 1A). At E12.5 this expression is expanded (Figures 1B–1F), but within the ventricular zone occurs mainly in radially oriented stripes that may represent clones of cells that recombined the GFP reporter. Although the ZEG reporter (Novak et al., 2000) underrepresents Cre+ cells, (Figures 1E and 1F; see also Xu et al., 2008), immunofluorescence detection of Cre also reveals a mosaic expression of Cre relative to all cells in the MGE ventricular zone (Figure 1D).

**Reduced NKKX2.1 and Upregulated Gsx2 Expression in the MGE of Six3Cre;Smo0/° Embryos**

Our previous study found that SHH signaling reversibly maintains NKKX2.1 expression and interneuron fate determination by mitotic progenitors in the MGE (Xu et al., 2005). To evaluate the effects of mosaic inactivation of SHH signaling on cell fate within the MGE, and to extend the fate analysis to older ages in vivo, Six3Cre mice were mated to a loxP-flanked Smoothened (Smo0/°) line (Dassule et al., 2000). The Six3Cre;Smo0/° mutants develop and survive grossly normally. At E14.5, Six3Cre;Smo0/° mutants have a patchy reduction of NKKX2.1 expression in the MGE (Figures 2C’ and 2G’). Colabeling with the S-phase marker BrdU administered 1 hr prior to embryo fixation revealed that elimination of SHH signaling results in the abnormal presence of non-NKKX2.1 expressing S-phase nuclei in the MGE (Figures 2D and 2D’).

The reduction of NKKX2.1 in the MGE of Six3Cre;Smo0/° mutants raises the question of whether these progenitors have been converted into an alternative telencephalic fate. Based on the general function of SHH to permit ventral patterning in the telencephalon (Ericson et al., 1995; Rallu et al., 2002; Shimamura and Rubenstein, 1997), we hypothesized that more dorsal pallidal fates (LGE and CGE) may be produced by the Smo-independent progenitors. Gsx2 is a homeodomain transcription factor that is normally enriched in the dorsal region of the lateral ganglionic eminence (LGE), and in the caudal ganglionic eminence (CGE, see Figure S1A available online) (Corbin et al., 2003; Hsieh-Li et al., 1995). In Six3Cre;Smo0/° mutants, there is a prominent upregulation of Gsx2 in the MGE (Figure 2E’). Like the reduced expression of NKKX2.1, upregulation of Gsx2 occurs in a patchy or striped pattern, and colabeling for both proteins reveals a complimentary pattern of expression of NKKX2.1 and Gsx2 in these mutants (Figure 2H’). Interestingly, the MGE-like region...
of Nkx2.1 nulls also shows an increase of GSX2 expression at E13.5 (Figure S1D). In sum, mosaic reduction of SHH signaling in the MGE progenitors results in downregulation of Nkx2.1, and increased expression of GSX2.

To determine whether this phenotype is due to repatterning of Nkx2.1-expressing progenitors, or to ectopic generation of Six3 from the mutant (Six3;Smo+/−;ZEG), nor is there detectable upregulation of GSX2 (arrows in F−H; Nkx2.1 and GSX2 merged image in H and H'). Scale bar represents 200 μm in (A) and applies to (B−)C', (E), and (E'), 100 μm in (D) and applies to (D'), and 10 μm in (F) and applies to (F')−H′.

Reduced Detection of PV+ Interneurons in the Postnatal Cortex of Six3Cre;Smo+/− Mutants

In mice, most cortical interneurons (about 80%, see Tamamaki et al., 2003) express either the calcium binding protein parvalbumin (PV), the neuropeptide somatostatin (SST), or the calcium binding protein calretinin (Calr). These groups are nonoverlapping, with the exception of a minority of Calr+ multipolar interneurons that coexpress SST (Xu et al., 2006). A variety of genetic fate mapping and transplantation studies have suggested that PV+ and SST+ interneurons originate predominantly within the Nkx2.1-expression domain of the MGE and ventral caudal ganglionic eminence (CGE), whereas most bipolar, Calr+ interneurons originate in the dorsal CGE (Butt et al., 2005; Fogarty et al., 2007; Xu et al., 2004, 2008). In the Six3Cre;Smo+/− mutants, the reduction in the MGE expression of Nkx2.1, which is required for the specification of both the PV+ and the SST+ subgroups (Du et al., 2008; Xu et al., 2004), suggests that both of these subgroups will be reduced in postnatal brains of these mutants. Although the cortical lamination is well preserved by NeuN and TBR1 staining (Figures S2I−S2J), only PV and the general interneuron marker GABA are significantly reduced in these mutants (Figures 3A−3D) at postnatal day 25 (P25). In contrast, the detection of the SST+ interneurons, as well as the partially overlapping NPY-expressing subgroup, is surprisingly unchanged.

Our previous study suggested that Nkx2.1 expression, maintained by SHH signaling, is required during the final cell cycle for the specification of MGE-derived interneurons (Xu et al., 2005).
We crossed Six3Cre;Smof/+ and ZEG Cre reporter lines to examine cell-autonomous fate effects of disruptions of SHH signaling (Figures 3E–3F). Although the ZEG line underreports Cre-expressing cells (Figure 1; Xu et al., 2008), analysis of Six3Cre;Smof/+;ZEG mutants revealed a significant reduction of the density of GFP+ interneurons that express either PV or SST (Figure 3G; for each comparison N = 6, p < 0.03 by two-tailed Wilcoxon signed rank test).

Because analysis of the same brains revealed that the total density is not decreased for the SST+ subgroup (Figure 3D), the mutant Six3Cre;Smof/+;ZEG cortex contains a higher proportion of SST+ cells that are GFP negative than controls. In light of previous evidence that Smo inactivation in postmitotic SST+ interneurons does not alter their migration or survival (Xu et al., 2005), this result suggests there is increased production of SST+ interneurons by Cre-negative progenitors within the
MGE. To directly examine this possibility, we took advantage of the fact that ZEG mice contain a dual-reporter system whereby cells express β-galactosidase (β-Gal) unless there is Cre-mediated conversion to GFP expression (Novak et al., 2000). Because we were unable to obtain unequivocal localization of β-Gal signal to distinct neurons in tissue sections (where all non-Cre-expressing lineages are β-Gal+), we plated dissociated cells from the MGE of Six3Cre;Smo0/−;ZEG mutant and Six3Cre;Smo0/+;ZEG control embryos on feeder cultures made from neonatal, nontransgenic mice (Figure 4A) (Xu et al., 2004). After 13 days in vitro (DIV), samples from the mutants have a dramatic increase in the percentage β-Gal+ cells that colabel with SST (Figures 4B–4H; 46.3% ± 3.1 versus 20.1% ± 1.1 p < 0.01, N = 3). These result strongly suggest that the normal density of SST+ interneurons in the Six3Cre;Smo0/+ cortex occurs due to the reduction in SST+ interneurons from progenitors that inactivated Smo, together with a compensatory increase in SST+ interneurons derived from progenitors that did not recombine Smo.

Upregulation of SHH Signaling in Cre-Negative MGE Progenitors of Six3Cre;Smo0/− Mutants

The surprising compensatory production of SST+ interneurons by the mutant MGE led to the re-examination of patterning in this region. Recent studies have suggested that most SST+ interneurons originate in the dorsal MGE (dMGE), and there is a complimentary bias for PV+ interneurons to originate in the ventral MGE (Flames et al., 2007; Fogarty et al., 2007; Wonders et al., 2008). The dMGE expresses high levels of SHH signaling effector Gli1 (Figure 4I), as well as Nkx6.2 (Figure 4K), a transcription factor whose expression in this region is SHH dependent (Xu et al., 2005). The dMGE also expresses increased levels of Hedgehog interacting protein-1 (Hhip1) and the SHH receptor Patched-1 (Ptc1; Figure 4J), two additional genes whose expression tends to be enhanced in regions of high levels of SHH signaling (Chuang et al., 2003). Analysis of the MGE of Six3Cre;Smo0/+ mutants at E14.5 reveals a dramatic upregulation of Gli1, Ptc1, and Nkx6.2 mRNA expression in the ventricular zone of middle and ventral regions of the MGE (Figures 4I–4K).

To determine whether this upregulation is dependent on the mosaic expression of Cre, communofluorescence for Cre and Ptc1 was conducted. In sections from middle or ventral regions of the MGE from Six3Cre;Smo0/+ heterozygous control embryos, signal for Ptc1 is weak and has a fairly uniform pattern along the ventricular surface (Figure S3C). In contrast, in the MGE of Six3Cre;Smo0/+ embryos, Ptc1 is strongly expressed in patches of MGE cells (Figures S3C–S3D). Remarkably, cells with higher Ptc1 signal are predominantly those with little or no signal for Cre (Figures S3B–S3D). Co-labeling of Nkx6.2 and Cre provides very similar results (Figures S3E–S3H). Together with the ectopic enrichment of GSX2 in the Nkx2.1-downregulated patches (Figure 2), these results suggest that within-MGE patterning of the Six3Cre;Smo0/+ reflects a mosaic of cell-autonomous and cell-nonautonomous effects. Progenitors that downregulate SHH signaling reduce Nkx2.1 expression and upregulate GSX2. In contrast, surrounding progenitors that have not inactivated Smo respond by upregulating SHH signaling and other transcripts normally enriched in the dMGE. Accordingly, there is a cell-nonautonomous expansion of SST production by MGE progenitors in this mutant (Figure 4H).

These results also suggest that relative to PV fate determination, higher levels of SHH signaling direct the specification of the SST fate. To determine whether exogenous SHH ligand can enhance the specification of SST+ interneurons by progenitors of ventral regions of the MGE, 10 nM SHH ligand was added to telencephalic slices at E11.5 from pan-GFP expressing embryos (Figure 5). Our previous study found that SHH addition to forebrain slices from Shh conditional null mice can rescue Nkx2.1 expression and interneuron fate, but only in progenitors that proliferated after being re-exposed to SHH (Xu et al., 2005). In order to limit the current analysis to cells that proliferated after exposure to exogenous SHH, retrovirus carrying an expression cassette for lacZ was added to the culture medium during the second DIV. After 2 days, the ventral half of the MGE was dissociated and transplanted into the neonatal neocortex (Figure 5A), an approach we and others have used extensively to study interneuron fate determination (Cobos et al., 2005; Du et al., 2008; Wonders et al., 2008). Thirty days after transplantation, analysis of tissue sections from controls (no exogenous SHH) revealed the expected bias for PV-expressing interneurons over those that express SST (Figure 5L). Remarkably, exogenous SHH resulted in a reversal in the proportion of PV+ versus SST+ interneurons, with a roughly 2-fold increase in SST+ cells in transplants from SHH-treated slices (Figure 5L; p < 0.01, N = 4 for both the PV decrease and SST increase). In contrast, the percentage of GFP+ cells that colabeled with β-Gal+ did not differ between conditions, suggesting that SHH treatment did not grossly alter the proliferation of interneuron progenitors.

Smo-inactivated MGE Progenitors Can Generate Bipolar Calr+ Interneurons

Smo-inactivated progenitors appear to upregulate GSX2 (Figure 2). This transcription factor is expressed weakly in MGE progenitors, and is enriched within the dorsal region of the LGE as well as in the CGE, a region that gives rise to vertically oriented, bipolar or bitufted Calr+ interneurons (Butt et al., 2005; Xu et al., 2004). As expected, in Six3Cre;Smo0/+;ZEG controls essentially no such GFP+, bipolar calretinin-labeled interneurons are present (Figures 6A–6C), although a separate, MGE-derived subgroup with multipolar morphology that generally coexpress SST are detected (Figure 6; see Xu et al., 2006). However, the Six3Cre;Smo0/+;ZEG mutant cortex contains the remarkable appearance of GFP+, vertically oriented, bipolar or bitufted Calr+ interneurons (3.8% ± 0.6 versus 0.5% ± 0.2 in Figures 6D–6G; p < 0.003, N = 5). These cells do not express SST (Figures 6E, 6F), further suggesting that they represent the aberrant production of a CGE-interneuron fate by MGE progenitors in the context of reduced SHH signaling. A similar result has recently been reported in conditional nulls for Nkx2.1 (Butt et al., 2008). These results suggest that during the age range of neurogenesis, SHH maintains MGE identity generally (Xu et al., 2005), while specifying MGE-specific subgroups through differential levels of signaling.
Regulation of Bipolar Calr+ Interneuron Fate by GSX2

GSX2 is strongly expressed in the lateral and caudal ganglionic eminence, and functions in retinoid production and patterning in the developing striatum (Corbin et al., 2000, 2003; Toresson et al., 2000; Waclaw et al., 2004; Yun et al., 2001). To determine whether GSX2 also plays an instructive role in the specification of...
bipolar Calr+ interneurons, Gsx2 was overexpressed by electroporation of the MGE of E12.5 slice cultures (Figures 7A–7H). After one DIV, cells were collected, plated onto neonatal cortical feeder cultures, and examined after 13 DIV. Compared with controls, expression of GSX2 in MGE progenitors roughly tripled the percentage of transfected cells that differentiate into Calr+, bipolar cells (pCAGIG control: 3.7% ± 1.3; pCAGIG-Gsx2: 10.3% ± 1.4, p < 0.005, N = 6).

To determine whether Gsx2 is required for the generation of normal numbers of bipolar, Calr+ interneurons in vivo, Gsx2 conditional null mutants (cKOs) were examined. In these mutants, Gsx2 expression is eliminated in the vast majority of telencephalic progenitors by approximately E12.5 (Figures S1A, S1B). Moreover, these mutants show similar dorsal-ventral patterning defects in the telencephalon as that previously reported in germ-line mutants (data not shown). In adult somatosensory cortex, there was a dramatic loss of bipolar Calr+/SST- negative interneurons in the Gsx2 cKOs (67.9 ± 5.9/mm² versus 112.6 ± 12.4/mm², p < 0.048 for layers 2–4, and 12.6 ± 2.3/mm² versus 19.1 ± 1.7/mm², p < 0.043 for layers 5 and 6, N = 3, Figures 7I–7M). In contrast the density of Calr+/SST+ interneurons (dorsal-MGE derived) was not significantly affected but showed a trend toward increase in the mutant (53.7 ± 6.4/mm² versus 47.0 ± 6.5/mm² for layers 2–4, N = 3, N.S.; and 16.8 ± 1.7/mm² versus 14.6 ± 1.6/mm² for layers 5 and 6, N.S., n = 3). In sum, loss of Gsx2 in telencephalic progenitors results in a selective reduction of the CGE-derived vertically oriented Calr+ population, but has little or no affect on the multipolar Calr+ population that coexpresses SST and originates in the MGE.

DISCUSSION

Although recent years have seen tremendous advances in understanding neural subtype fate determination in the mammalian nervous system, such advances in forebrain development have lagged, despite the relevance to neuropsychiatric disease. In this paper, we provide inroads into the fate determination of the three main neurochemically defined subgroups of cortical interneurons.
interneurons: those that express PV, SST, or Calr. The PV- and SST-expressing subgroups have previously been shown to require NKX2.1 function for their fate determination (Butt et al., 2008; Xu et al., 2004), and this function requires the maintenance of NKX2.1 expression in the MGE progenitors by SHH signaling (Xu et al., 2005). Here we provide evidence that SHH signaling also regulates intra-MGE patterning and the differential specification of PV+ versus SST+ interneurons. In addition, this signaling represses GSX2 expression, preventing the re specification of some MGE progenitors to the bipolar Calr+ subgroup. These results demonstrate that cortical interneuron diversity is critically influenced by differential levels of SHH signaling within the ventral telencephalon. Moreover, the pronounced cell-nonautonomous effects of mosaic Smo inactivation reveal the remarkable, albeit fate-restricted, plasticity of MGE progenitors during the period of neurogenesis.

Shifts in MGE Patterning and Fate Produced by Mosaic Alterations of SHH Signaling

Over the previous decade evidence has been accruing that, like the developing spinal cord, in the telencephalon much of the regulation of neuronal fate determination occurs by the production of neuronal subtypes in molecularly distinct progenitor domains (Flames et al., 2007; Lupo et al., 2006). Recently, progenitor subdomains have been identified within the MGE, with the dorsal MGE expressing higher levels of Gil1, Ptc1, Hhip1, and Nkx6.2 (see Figure 4 and Xu et al., 2005; Flames et al., 2007; Wonders et al., 2008). Gil1, Ptc1, and Hhip1 detectability are generally indicative of high levels of SHH signaling (Chuang et al., 2003; Fuccillo et al., 2006), and the dorsal MGE domain of Nkx6.2 and Gil1 expression are abolished in NestinCre;Shh f/f mutants (Xu et al., 2005). In this context, it is initially surprising to find that Six3Cre;Smo f/f mutants show strong upregulation of Gil1, Ptc1, and Nkx6.2 in strips of progenitors of the middle and ventral MGE (Figure 5). However, Cre and PTC1 coimmunodetection suggests that the cells expressing higher signal for PTC1 are those with lower signal (if any) for Cre (Figure S3). In Drosophila, loss of Hedgehog (Hh) receptivity results in higher levels of Hh signaling in surrounding cells, due to the action of Ptc to sequester Hh protein (Chen and Struhl, 1996). A similar phenomenon in the developing diencephalon has been reported with conditional nulls of Smo (Machold et al., 2003). However, from the current analysis it remains unclear whether reduced sequestration of SHH by the Smo-recombined cells, or some other mechanism accounts for the dramatic cell-nonautonomous effects seen. Both analyses of tissue sections from Six3Cre;Smo f/f mutants (Figure 3), and culture experiments that distinguish the fates of
recombined or nonrecombined progenitors from these mutants (Figure 4), indicate that these patterning changes produce both cell autonomous and nonautonomous alterations in interneuron fate. Thus, mosaic inactivation of Smo in the MGE results in dramatic cell-autonomous (reduction of NKX2.1 and upregulation of GSX2; Figure 2) and cell-nonautonomous (upregulation of SHH responsive genes including Nkx6.2; Figure 4) effects on patterning and fate determination within the MGE (Figure 8). This is a rare example in which cell-nonautonomous effects on neuronal fate determination have been demonstrated to the level of fully differentiated neurons in the mammalian forebrain.

Figure 7. GSX2 Functions in the Generation of Bipolar Calretinin-Expressing Interneurons

(A–H) Results from a Gsx2 gain-of-function study. (A) E12.5 MGE progenitors were electroporated with pCAGIG (control, B–D) or pCAGIG-Gsx2 (E–G) plasmid. After 1 day, the MGE was dissociated and plated onto neonatal cortical cells and cultured for 13 days. GFP labels transfected cells. Image (B) and (C) are merged in (D). (E) and (F) are merged in (G). In controls, colabeling of Calr and SST is present in cells with multipolar morphology (arrow), but Calr labeling in bipolar neurons is rare. In contrast, transfection of pCAGIG-Gsx2 results in a more than 2-fold increase in the percentage of GFP+ cells with a Calr+, bipolar phenotype (arrowhead in E and G; quantified in H; n = 6). These cells do not express SST (F).

(I–M) In the lower panel, the density of Calr+ interneurons in somatosensory cortex of conditional Gsx2 mutant mice (cKO; Foxg1-tTA;Tet-Cre;Gsx2f/f, K and L) are compared with the Cre-negative controls (I and J). Arrowheads indicate the vertically oriented Calr+, SST-negative interneurons. (M) Gsx2 cKO mice show a significant reduction of Calr+, SST-negative interneurons in both upper and lower layers (n = 3). The relative density of PV+ or SST+ interneuron subgroups is not affected (data not shown).

Scale bar represents 25 μm in (B)–(G), and 50 μm in (I) and applies to (J)–(L).

Although the results presented make the case that SHH signaling participates in intra-MGE patterning and the differential fate determination of interneuron subgroups, a number of interesting questions remain. First, given the expression domains of Shh mRNA within the ventral telencephalon, in the ventricular zone of the preoptic region and mantle zone of the MGE, it is surprising that SHH signaling would be strongest within dorsal-most MGE. However, at E12.5 Shh mRNA is strongly expressed in both dMGE and vMGE mantle (for example, see Figure 2O in Gulacsi and Anderson, 2008). The dMGE SHH signaling enrichment suggests that SHH concentration is distorted by variable diffusion within the extracellular matrix or intercellular transport, and that another signaling pathway, such as that of fibroblast growth factors (FGFs), might be relatively more important for ventral MGE expression of interneuron fate determining genes (Gutin et al., 2006; Hebert and Fishell, 2008; Storm et al., 2006). In addition, because different interneuron subtypes are generated at different times (Butt et al., 2005, 2008), both the level of SHH signaling and its duration are likely to be critical (Ahn and Joyner, 2004; Dessaud et al., 2007; Harfe et al., 2004).

**Gsx2 Functions in Interneuron Fate Determination**

Other questions raised by these results pertain to the pronounced upregulation of GSX2 in recombined MGE progenitors of Six3Cre;Smo<sup>fl</sup>f<sup>mt</sup> mutants. GSX2 is expressed throughout the pallidum but is enriched in the more dorsal regions of both the lateral and the caudal ganglionic eminences (Corbin et al., 2003; Yun et al., 2001). The upregulation of GSX2 in Smo-recombinant cells that also downregulate NKX2.1 (Figure 2) raises the possibility that NKX2.1 normally represses GSX2 expression. Evidence for increased GSX2 in the pallidum of Nkx2.1 nulls at E13.5 supports this contention (Figures S1C–S1D). However, this effect must be both partial and stage dependent, because GSX2 is expressed weakly in the MGE and ventral expansion of high levels of GSX2 expression in Nkx2.1 nulls is not seen at earlier ages (Corbin et al., 2003). Because Nkx2.1 mutants also have a dramatic reduction of Shh expression secondary to the loss of MGE mantle zone (Sussel et al., 1999), the later upregulation of GSX2 in these mutants, and the upregulation of GSX2 in the Six3Cre;Smo<sup>fl</sup>f<sup>mt</sup> mutants, may reflect the loss of NKX2.1 in a very low-SHH signaling context.

A related issue regarding the upregulation of NKX2.1 expression in Six3Cre;Smo<sup>fl</sup>f<sup>mt</sup> mutants is the fates of these progenitors. The cortex of Six3Cre;Smo<sup>fl</sup>f<sup>mt</sup>ZEG mice show a dramatic increase, from a control level of essentially none, of vertically oriented, bipolar or bitufted, Calr<sup>+</sup> cells from Cre<sup>+</sup> progenitors (Figure 6). In the cortex of adult Cre-reporter transgenic mice, these interneurons are labeled by Gsx2Cre but not by Nkx2.1Cre or Nkx6.2Cre (Fogarty et al., 2007; Xu et al., 2008). These data, transplantation and culture studies (Butt et al., 2005; Xu et al., 2004), and evidence that their specification does not require NKX2.1 (Xu et al., 2004), suggest that the bipolar Calr<sup>+</sup> population primarily originates from GSX2+ progenitors in the CGE. The presence of GFP<sup>+</sup>, bipolar Calr<sup>+</sup> and SST-negative cells in the cortex of Six3Cre;Smo<sup>fl</sup>f<sup>mt</sup>ZEG mice thus suggests that downregulation of SHH signaling converts some MGE progenitors into CGE-like fates. Our gain and loss of function studies further

**SHH Signaling Defines Interneuron Subgroup Fates**

**Figure 8. Differential Specification of Cortical Interneurons by SHH Signaling and the Homeomain Transcription Factors NKX2.1 and GSX2**

(A) Schema of NKX2.1 (purple), NKX6.2 (green), and GSX2 (blue) expression in the pallidal telencephalon at E13.5. In controls, GSX2 is expressed at high levels in the dorsal region of the pallidal LGE and CGE, and weakly through the MGE. NKX6.2 is expressed along the MGE-LGE sulcus and dorsal-most MGE (dMGE), whereas NKX2.1 is expressed throughout the MGE.

(B) Relationship of primary origin to interneuron fate. Most SST<sup>+</sup> interneurons originate in the dorsal MGE, whereas most PV<sup>+</sup> interneurons have a ventral MGE (vMGE) origin. Most bipolar, Calr<sup>+</sup> interneurons originate from the CGE (not shown). In the Six3Cre;Smo<sup>fl</sup>f<sup>mt</sup> mutant, mosaic loss of SHH signaling results in cell-autonomous loss of NKX2.1 expression in the MGE, and some of these cells upregulate GSX2. This conversion is associated with the abnormal production of bipolar, Calr<sup>+</sup> interneurons from Six3-lineage MGE progenitors. At the same time, Cre-negative progenitors in the vMGE upregulate SHH signaling and Nkx2.2, converting their production to SST<sup>+</sup> interneurons.

(C) A two-state model for the role of SHH on interneuron fate determination in the MGE, after initial patterning has been established. High levels of SHH signaling in the dMGE drive NKX2.1<sup>+</sup> progenitors to produce mainly SST<sup>+</sup> interneurons, while lower levels of SHH signaling in the vMGE maintains the generation of the PV<sup>+</sup> subgroup. On the other hand, bipolar Calr<sup>+</sup> interneurons are generated by high GSX2<sup>+</sup> progenitors in the CGE, that at this stage have become independent of SHH signaling (MacEwan et al., 2003).
suggest that Gsx2 contributes to this respecification event (Figure 7).

**A Model of SHH-Controlled Interneuron Genesis**

In the telencephalon, as in the spinal cord, SHH signaling contributes to the establishment of dorsal-ventral patterning primarily by preventing the formation of truncated, "repressor" form of GLI3 (Litingtung and Chiang, 2000; Rallu et al., 2002). Although the decision of which telencephalic tissue will be pallial (cortical) or pallidal (subcortical) appears to be insensitive to alterations of SHH signaling by E11.5 (Machold et al., 2003; Rallu et al., 2002), neuronal fate determination within the patterned pallidum retains a remarkable degree of plasticity into the age range of neurogenesis. For example, exogenous SHH applied to slices from NestinCre;Shh<sup>+</sup> nulls can rescue both the expression of Nkx2.1 in MGE progenitors, and the SST+ differentiated fates of cells that cycled after re-exposure to SHH (Xu et al., 2005). Moreover, conditional loss of Nkx2.1 expression at E12.5 results in the conversion of some MGE progenitors from the generation of SST+ to the generation of bipolar Calr+ subgroups (Butt et al., 2008). Taken together with the results of this study, these results suggest a model whereby Nkx2.1-expressing progenitors retain plasticity for the generation of SST- versus PV-expressing subgroups, responding to high levels of SHH signaling by primarily generating SST+ interneurons, and responding to lower levels of SHH signaling by primarily generating PV+ interneurons (Figure 8).

In sum, we provide evidence that SHH signaling is critical to both the maintenance of MGE-derived interneuron progenitor fates, and to the differential specification of two major subgroups of cortical interneurons. Mosaic loss of SHH signaling within MGE progenitors reveals a remarkable degree of progenitor plasticity resulting in both cell-autonomous and cell-nonautonomous changes in intra-MGE patterning and interneuron fate. Moreover, these mice have a pronounced increase in cortical interneurons. Mosaic loss of SHH signaling by E11.5 (Machold et al., 2003; Rallu et al., 2002). Slices were maintained in Nb/B27 for 20 hr with no change of medium. The recombinant mouse Sonic Hedgehog N-terminus (C25II, R & D Systems, catalog number: G507A) for 48 hr with no change of medium. The recombinant mouse Sonic Hedgehog N-terminus (C25II, R & D Systems, catalog number: G507A) for 48 hr with no change of medium.

**Tissue Preparation**

Embryonic heads were immersion-fixed for 2 hr in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) containing 6% sucrose. Brains older than E12.5 were dissected from the head for fixation, cryoprotected with increasing grades of sucrose (15%–30%) in 0.1 M phosphate buffer (PB, pH 7.4), embedded in OCT Compound (Tissue-Tek), and stored at −80°C. Sections were cut with a cryostat (Leica) at 12 μm thickness.

Postnatal mice were perfused with PBS followed by 4% PFA with 0.0125% glutaraldehyde in PBS. Brains were postfixed in the same fixative in 60 mM PB overnight at 4°C. For sectioning, brains were embedded in 4% low-melting-point agarose and cut in coronal plane at 40 μm thickness with a vibratome (Leica). Sections were stored in antifreeze solution (30% glycerol and 30% ethylene glycol in 40 mM PB, pH 7.4) at −20°C.

**Telencephalic Slice Cultures**

Coronal telencephalic slices, 250 μm thick, were prepared and cultured as described elsewhere (Xu et al., 2004). Slices were maintained in Neurobasal/B27 (NB/B27; Gibco) with 5 ng/ml recombinant human FGF2 (Promega, catalog number: G507A) for 48 hr with no change of medium. The recombinant mouse Sonic Hedgehog N-terminus (C25II, R & D Systems, catalog number: G507A) for 48 hr with no change of medium. The recombinant mouse Sonic Hedgehog N-terminus (C25II, R & D Systems, catalog number: G507A) for 48 hr with no change of medium. The recombinant mouse Sonic Hedgehog N-terminus (C25II, R & D Systems, catalog number: G507A) for 48 hr with no change of medium.

**Cocultures of MGE-Derived Cells on Cortical Feeder Cells**

The cortical feeder cultures (100,000 cells per 36 mm<sup>2</sup> well of 16-well chamber slides, Lab-Tek<sup>®</sup>) were prepared from the dissociated cortices of neonatal pups as described previously (Xu et al., 2004). For the Six3Cre;cma<sup>-</sup>/<sup>-</sup>;ZEG mutant and Six3Cre;cma<sup>-</sup>/<sup>-</sup>;ZEG control coculture experiment, the E14.5 embryos were genotyped by polymerase chain reaction and green fluorescent protein expression. The MGE was dissected from the brain slices and then dissociated, gently triturated, and resuspended in Nb/B27 medium. Five thousand cells per well were added to cortical feeder cultures prepared 3 to 5 days earlier. All cultures were maintained and processed as described elsewhere (Xu et al., 2004).

**Neonatal Transplantation**

Transplant cells were suspended in 10–15 μl Nb/B27 medium to a cell concentration of roughly 20 cells/μl. The cells were filled from the rear end of a pulled glass micropipette (0.5 mm I.D., 1 mm O.D.), and then fitted to the nanoinjector (Nanoject II, Drummond). P1 neonate pup was anesthetized in ice for 3 min before the injection. Each pup received 30 injections of 69 nl into each of the two hemispheres. The injection sites are in the middle between bregma and interaural line, 1 mm lateral to the midline (Figure 5). The micropipette tip was placed 1 mm deep to the pial surface, allowing for the injection of cells mainly into layers 3–5.

**Slices Electroporation**

Mouse Gsx2<sup>−</sup>CDNA was subcloned into pCAG-Gsx2-IRES-GFP, abbreviated as pCAGI-G-Gsx2. The same vector without Gsx2<sup>−</sup> was used as control. DNA was purified with the Endofree Plasmid Maxi Kit (Qiagen) and electroporated into the MGE region of E12.5 slices as described previously (Stuhmer et al., 2002). Slices were maintained in Nb/B27 for 20 hr with no change of medium. Then the MGE was dissociated and cultured as above.
**In Situ Hybridization and Immunohistochemistry**

Nonradioactive DIG-labeled probes were used for in situ hybridization as described previously (Xu et al., 2005). The cDNA probes used were Shh (BC063087 from Open Biosystems), GIL1 (Kinzler et al., 1988), Ptc1h, Nkx6.2 (gift from Dr. Gerd Fishell).

The primary antibodies for immunofluorescence labeling included anti-BrdU (rat, Serotec, 1:200), calretinin (rabbit, Chemicon, 1:2000; mouse, Swant, 1:5000), calbindin (rabbit, Swant, 1:5000), Cre (mouse, Chemicon, 1:1000), GABA (rabbit, Sigma, 1:5000), GFP (rabbit, Molecular Probes; or chick, Abcam, all 1:2000), GSK2 (rabbit, made by Kenneth Campbell, 1:5000), NeuN (mouse, Chemicon, 1:1000), Nkx2.1 (rabbit, Abcam, 1:1000; mouse, Neomarkers, 1:100), Nkx6.2 (rabbit, a kind gift from Maike Sander, 1:1000), NPY (rabbit, Immunostar, 1:2000), somatostatin (rat; Chemicon; 1:400), parvalbumin (mouse; Chemicon; 1:5000), PTH1 (rabbit, Santa Cruz Biotechnology, 1:500), and TBR1 (rabbit, a kind gift from Robert Hever, 1:1000). Fluorescent secondary antibodies were Alexa Fluorophore (Molecular Probes, 1:500). For triple labeling, an additional Cy5-conjugated secondary (Jackson Immunoresearch) was used. DAPI (300 nM) was applied together with the secondary antibodies as a nuclear marker.

Immunostaining was performed as described previously (Xu et al., 2005). For detection of Cre, Nkx2.1, GSK2, TBR1, and NeuN sections were treated with 1 mM EDTA at 65°C for 10 min. Sections aged 12.5 or younger were first fixed with 4% PFA in PBS at room temperature for 10 min to prevent them from peeling off the glass during EDTA treatment.

**Imaging and Cell Counting**

Fluorescent and bright field images were collected on a Nikon E800 microscope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging). Confocal images were obtained on ZEISS LSM scope equipped with a Cool SNAP HQ (Photometrics) camera and MetaMorph software (Universal Imaging).

For each postnatal brain, interneuron population size was analyzed preferentially on the right hemisphere of somatosensory cortex from three coronal sections that were 400 μm apart, located rostral to the genu of the corpus callosum and caudal to the hippocampal commissure. Three to six brains were examined for one group. Cortical laminar position was determined by cell packing density with DAPI staining, and separate counts were tabulated. A mouse atlas (Franklin and Paxinos, 1997) was referenced for determining laminar and area location.

Stereological counting of neurochemically defined interneuron subgroups was conducted on a Nikon E600 microscope fitted with Stereo Investigator software (MicroBrightField, Inc.) with the fractionator or optical fractionator. Stereological analyses were present as average and standard error of the mean (± SEM) and carried on Excel (Microsoft Office) for Student’s t test (two tailed, unpaired), and on Prism 4 (GraphPad) for Wilcoxon signed rank tests.

**References**


**Supplemental Information**

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.neuron.2010.01.004.

**Acknowledgments**

This work was supported by P01 NS048120 (S.A.A., H.M.), R01 MH069612 and K02 MH070031 (S.A.A.), R01 NS044080 (K.C.) and NARSAD (O.J.X.). The authors wish to thank Karin H. Krueger and Ping Song for excellent technical assistance.

Accepted: January 4, 2010
Published: February 10, 2010


Sonic Hedgehog Signaling Confers Ventral Telencephalic Progenitors with Distinct Cortical Interneuron Fates

Qing Xu, Lihua Guo, Holly Moore, Ronald R. Waclaw, Kenneth Campbell, and Stewart A. Anderson
Supplemental Figure S1. Related to Figure 2. Changes of GSX2 expression in the ganglionic eminences of mutant mice.

(A-B) Loss of GSX2 expression in the caudal ganglionic eminence (CGE) of Foxg1-tTA;Tet-Cre;Gsx2f/f mutants. Shown is GSX2 immunofluorescent signal in coronal cryosections through the CGE from E12.5 embryos. As is the case in more rostral sections through the telencephalon, in the control section (A) GSX2 is expressed in a dorsal pallidum high to ventral pallidum low gradient. In contrast, the mutant section (B) shows only sporadic expression of GSX2 within the CGE. As expected with the Foxg1-tTA;Tet-Cre system, GSX2 is expressed in the diencephalon (arrows, ventral thalamus, VT). The reduction of GSX2 within the CGE of these mutants is consistent with the dramatic reduction of vertically oriented, bipolar, Calr+ interneurons (Fig. 7) that are known to arise within this domain (Butt et al., 2005, Xu et al., 2004).

(C-D) Upregulation of GSX2 into the ventral pallidum of E13.5 Nkx2.1-/- mutants. As shown in Fig. 2, GSX2 expression appears to be upregulated within Smo-recombined cells in the MGE of Six3Cre;Smof/f mutants, and NKX2.1 is downregulated in these same progenitors. To examine the possibility that the loss of NKX2.1 expression itself contributes to the de-repression of GSX2 in these cells, we examined GSX2 expression in the ventral pallidum, MGE-like region (MGE*) of Nkx2.1 mutants. At E9.5, GSX2 expression is not expanded in these mutants (Corbin et al., 2003). However, at E13.5, relative to the control embryo (C) that shows weak expression in the MGE, the Nkx2.1-/- embryo (D) appears to have upregulated GSX2 expression. This finding, together with previous studies on Nkx2.1 (Sussel et al., 1999, Xu et al., 2005) and evidence from conditional inactivation of Nkx2.1 in MGE progenitors (Butt et al., 2008) suggests that during the age range of neurogenesis NKX2.1 functions to maintain progenitor identity in the MGE.
Supplemental Figure S2. Related to Figure 3. In contrast to results at E14.5, SIX3Cre;Smo<sup>fl/fl</sup> mutants lack patterning changes at earlier ages.

(A-D') Lack of phenotype and co-expression of NKX2.1 with Cre in SIX3Cre;Smo<sup>fl/fl</sup> mutant and control embryos at the start of neurogenesis. Shown are 12 µm coronal cryosections through the telencephalon of a SIX3Cre;Smo<sup>fl/+</sup> control embryo (A-D) and a SIX3Cre;Smo<sup>fl/fl</sup> mutant (A'-D') at E10.5. (B, B') In contrast to the mutant embryos at E14.5 (Fig. 2), there are no NKX2.1-negative patches (DAPI+, NKX2.1(-) within the NKX2.1 expression domain in the ventral pallidal telencephalon. (C-D') In addition, nearly all Cre+ cells in both the mutant and control examples express NKX2.1. Similar results were seen in a total of 4 mutant and 4 control embryos. These results suggest that the patchy loss of NKX2.1 expression seen at E14.5 in SIX3Cre;Smo<sup>fl/fl</sup> mutants develops after the start of neurogenesis, and is likely to involve cells that initially expressed NKX2.1. Scale bar = 100 µm in (A) and applies to (A'), = 25 µm for (B-D').

(E-H') Lack of abnormalities in NKX2.1 or GSX2 expression in the MGE of SIX3Cre;Smo<sup>fl/fl</sup> mutant embryos at E12.5. Shown are two 12µm coronal cryosections through the MGE of a SIX3Cre;Smo<sup>fl/+;ZEG</sup> control embryo (E-H) and a SIX3Cre;Smo<sup>fl/fl;ZEG</sup> mutant (E'-H') at E12.5. Sections have been triple immunolabeled for NKX2.1, GFP, and GSX2 as indicated. (E, E') In contrast to the mutant embryos at E14.5 (Fig. 2), at E12.5 there are no obvious NKX2.1-negative patches in the mutant MGE. Panels (F, F') show the lack of clear loss of NKX2.1 expression occurs despite the strong expression of the recombination reporter GFP in the mutant MGE (F'). Panels (G, G') show that at E12.5 there is also no increased expression of GSX2 in the mutant MGE like that seen at E14.5. Taken together with the E10.5 data, these results suggest that the marked patterning alteration seen at E14.5 is a relatively late-developing phenotype. Scale bar = 50 µm in (E) and applies to (E'-H').

(I-J') SIX3Cre;Smo<sup>fl/fl</sup> mutants have normal lamination in the P25 cortex. Wild type (I, J) and mutant (I'-J') are compared on the immunofluorescence of NeuN (I-I', marker of most neurons),
TBR1 (J-J’, marker of most pyramidal neurons) that show overall normal distribution and density. Scale bar = 100 μm in (I) and applies to (I’-J’).
Supplemental Figure S3. Related to Figure 4. Cre-negative VZ progenitors up-regulate PTCH1 and NKX6.2 in the MGE of Six3Cre;Smo^{fl/+} mutants. Shown are photographs of the
MGE ventricular zone of coronal cryosections from control (A-D, E-H,Six3Cre;Smo$^{+/+}$) and mutant (A’-D’, E’-H’, Six3Cre;Smo$^{f/f}$) embryos aged E14.5. Boxes in (A) and (A’) indicate regions amplified in (B-D) and (B’-D’), respectively. Boxes in (E and E’) indicate regions of the MGE amplified in (F-H) and (F’-H’), respectively. Arrowheads in (C’) indicate regions of higher levels of signal for PTCH1 occur only in the mutant section, where they correspond to Cre-negative regions of the mutant ventricular zone (B’, D’). Arrows in (E and E’) indicate the sulci between the LGE and the MGE. Arrowheads in (F’-H’) indicate regions of higher levels of signal for NKX6.2 in the mutant sections, where they correspond to Cre-negative regions of the mutant ventricular zone (F’, H’). The complimentary expression patterns of Cre versus PTCH1 and NKX6.2, together with the expanded expression of Gli1, Ptc1 and Nkx6.2 mRNAs (Fig. 3. I-K’), suggest that upregulation of Shh signaling occurs in some progenitors that have intact Smo.

Scale bars = 100 μm in (A, A’, E, E’), =10 μm in (B-D’), = 25μm in (F-H’).