Spatial pattern of sonic hedgehog signaling through *Gli* genes during cerebellum development

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Summary

The cerebellum consists of a highly organized set of folia that are largely generated postnatally during expansion of the granule cell precursor (GCP) pool. Since the secreted factor sonic hedgehog (Shh) is expressed in Purkinje cells and functions as a GCP mitogen in vitro, it is possible that Shh influences foliation during cerebellum development by regulating the position and/or size of lobes. We studied how Shh and its transcriptional mediators, the Gli proteins, regulate GCP proliferation in vivo, and tested whether they influence foliation. We demonstrate that *Shh* expression correlates spatially and temporally with foliation. Expression of the Shh target gene *Gli1* is also highest in the anterior medial cerebellum, but is restricted to proliferating GCPs and Bergmann glia. By contrast, *Gli2* is expressed uniformly in all cells in the developing cerebellum except Purkinje cells and *Gli3* is broadly expressed along the anteroposterior axis. Whereas *Gli* mutants have a normal cerebellum, *Gli2* mutants have greatly reduced foliation at birth and a decrease in GCPs. In a complementary study using transgenic mice, we show that overexpressing Shh in the normal domain does not grossly alter the basic foliation pattern, but does lead to prolonged proliferation of GCPs and an increase in the overall size of the cerebellum. Taken together, these studies demonstrate that positive Shh signaling through Gli2 is required to generate a sufficient number of GCPs for proper lobe growth.

Key words: Shh, Foliation, Proliferation, Patterning

Introduction

Cerebellar development is a carefully orchestrated process that produces an exquisitely foliated structure with a simple layered cytoarchitecture. In mammals, the cerebellum is divided into three regions with distinct anteroposterior (AP) foliation patterns: a central vermis and two bilaterally symmetric hemispheres. The most abundant neurons in the cerebellum, as well as the entire brain, are the granule cells. Whereas Purkinje cells and cerebellar interneurons originate in the ventricular neuroepithelium, cerebellar granular cell precursors (GCPs) arise from a germinal zone in the rhombic lip situated in dorsal posterior rhombomere 1 (Altman and Bayer, 1997). The GCPs begin to leave the rhombic lip at approximately embryonic day (E) 13 and migrate over the cerebellar anlage to form the external granule layer (EGL). Although the EGL is formed by E15, GCPs in the EGL remain mitotically active until 2 weeks postnatal. Granule cells start to exit the cell cycle after birth and as part of their differentiation program migrate internally past the Purkinje cells to form the inner granule layer (IGL) (Wang and Zoghbi, 2001). Over the course of the first two postnatal weeks, cerebellar folia form, suggesting the increase in granule cells is largely responsible for foliation. The process of foliation begins with the formation of four principal fissures, which divide the cerebellum into five cardinal lobes (Altman and Bayer, 1997). As GCP proliferation continues, these lobes expand and are further subdivided to give rise to the species-specific foliation pattern observed in the mature cerebellum. The fissures that divide the central cardinal lobe into lobes VI-VIII are among the last to form in the vermis.

It has been shown that an interaction between Purkinje cells and GCPs is important for granule cell proliferation and foliation. For example, when Purkinje cells are ablated or in mouse mutants that lack Purkinje cells, such as *Lurcher* and *Staggerer*, the GCP population is diminished and foliation is arrested (Caddy and Biscoe, 1979; Herrup, 1983; Sidman et al., 1962; Smeyne et al., 1995; Wetts and Herrup, 1982). One key GCP mitogen expressed in Purkinje cells is sonic hedgehog (Shh), since it can induce proliferation of GCPs in culture, and injection of Shh antibodies into the cerebellum reduces granule cell proliferation (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Shh signaling is also involved in many other developmental processes, in particular in regulating cell fate decisions (Ingham and McMahon, 2001; Jacob and Briscoe, 2003). In the spinal cord Shh induces specific ventral cell types in a concentration-dependent manner, and in the limb Shh determines digit identity. Since

Shh mutants die at birth, we have utilized a gain-of-function approach to address the role of Shh in vivo during postnatal development in regulating GCP proliferation and a possible role in foliation.

Shh signaling is mediated by the Gli family of transcription factors. In the spinal cord Gli2 is the primary activator of Shh signaling, whereas Gli3 functions mainly as a repressor but is also a weak activator (Bai et al., 2002; Bai and Joyner, 2001; Bai et al., 2004; Persson et al., 2002). By contrast, in the limb only Gli3 is required for digit patterning and to regulate a normal level of proliferation (Litingtung et al., 2002; te Welscher et al., 2002; Wang et al., 2000). An important question, therefore, is whether Shh functions in the cerebellum primarily by inhibiting the Gli3 repressor as in the limb, and/or by inducing the activator Gli2. Due to the embryonic lethality of *Gli2* and *Gli3* mutants, the in vivo requirements for these two genes during postnatal cerebellum development have not been addressed. Gli1 (Gli – Mouse Genome Informatics), however, is not required for mouse development, although it plays a redundant activator function with Gli2, which is revealed only in *Gli2* heterozygotes (Bai et al., 2002; Park et al., 2000). Furthermore, unlike that of *Gli2* and *Gli3*, *Gli1* transcription is regulated by Shh signaling. In particular, all transcription of *Gli1* is absolutely dependent on induction of Gli2 and Gli3 activators by Hh signaling (Bai et al., 2004). Since *Gli1* is a transcriptional target of Shh signaling, *lacZ* expression in Gli-lacZ knock-in mice ($Gli1^{lz/+}$) is a readout of positive Shh signaling.

We utilized Gli1-lacZ mice to characterize the precise spatial and temporal pattern of positive Shh signaling in the developing cerebellum. Strikingly, Shh expression and signaling (Gli-lacZ expression) in the developing vermis is spatially patterned from E18 to P10 with highest levels in anterior lobes (III-VIa) and the most posterior lobe (X). Both Gli1 and Gli2 are primarily excluded from Purkinje cells, and Gli expression is strongest in Bergmann glia and in the GCPs in the outer layer of the EGL. *Gli3* is expressed in most cell types along the AP axis. We show that in the absence of *Gli2* normal expansion of GCPs in the EGL is impaired, and foliation is reduced at birth. Gli1-lacZ expression is undetectable in *Gli2* mutants, demonstrating that Gli2 is the major activator required to transduce Shh-positive signaling in the developing cerebellum. In support of this, the thickness of the EGL appears normal in *Gli3* mutants. In transgenic mice overexpressing *Shh* in a normal pattern in the cerebellum, the basic pattern of cerebellum foliation is maintained, although the entire cerebellum is enlarged and the lobes that normally express higher levels of Shh have an irregular IGL. In addition, the EGL persists longer than normal in transgenics. This study utilizes in vivo experiments to establish a role for positive Shh signaling in regulating expansion of the cerebellar lobes by regulating GCP proliferation, and demonstrates that Gli2 is a required mediator for this signaling.

Materials and methods

Mouse lines

Timing of embryos was determined by designating noon of the day a vaginal plug was detected as E0.5. The day of birth was designated as P0. Mouse lines were maintained on an outbred Swiss Webster background. *lacZ* knock-in mice $Gli1^{lz/+}$ and $Gli2^{lz/+}$ were genotyped by staining ear punches or tails in β-galactosidase (Bai et al., 2002; Bai and Joyner, 2001). *Shh-P1* mice were genotyped by PCR using primers 5′-GGTCGGCGACAACTCAATCG and 5′-GT-GAGGGTCTCTCAGCGTATG. Mice were genotyped for the $Gli1^{zfd}$ allele using PCR primers g1.5489 5′-TTGCAGCCAGGAGTTC-GATT, g1.6027R 5′-AGGACCCTACCTTGACTTGACACC and NeopmR 5′-AGACTGCCTTGGGAAAAGCG (provided by C. Bai). Mice were genotyped for the $Gli2^{zfd}$ allele as previously described (Mo et al., 1997).

β-galactosidase staining

Brains from P10 or later stages were dissected after intracardiac perfusion of mice with PBS followed by 4% paraformaldehyde. All brains were immersion fixed in 4% paraformaldehyde at 4°C for 30 minutes. Fixed tissue was cryoprotected in 30% sucrose overnight at 4°C and embedded in OCT (Tissue-Tek). β-gal activity was detected in 10-14 μm frozen sections by incubation in X-gal solution at 37°C for 4-6 hours unless otherwise indicated. Sections were counterstained in Nuclear Fast Red. Detailed protocols are available at http://saturn.med.nyu.edu/research/dg/joynerlab.

Histology, immunohistochemistry and RNA in-situ hybridization

Embryonic and early postnatal brains were dissected and immersion fixed in 4% paraformaldehyde overnight at 4°C. Brains collected after P5 were collected after intracardiac perfusion and fixed in 4% paraformaldehyde overnight at 4°C. Tissue was embedded in paraffin according to standard methods and sectioned at 5 μm. For consistency, sections analyzed from the vermis were limited to the most medial 100 μm. Histology was performed on paraffin sections using standard procedures. For antibody staining on X-gal stained sections, frozen sections were incubated in substrate for 2-4 hours and then post-fixed. Antibody staining was performed according to standard protocol. The following primary antibodies were used: BLBP (1:1000 kindly provided by N. Heintz), Calbindin (1:4000, Sigma), Calbindin (1:4000, Swant), and PCNA (1:500, Santa Cruz). Goat-anti-mouse and goat-anti-rabbit biotinylated secondary antibodies (Vector Laboratories) were used. Staining was visualized using an ABC kit (Vector Laboratories) and DAB substrate. RNA in-situ hybridization on sections was performed using standard methods. Detailed protocols are available at http://saturn.med.nyu.edu/research/dg/joynerlab.

Quantitation of external and inner granule layers

To quantify EGL thickness, high magnification images were taken of medial sections from wild-type (WT) and mutant cerebella of E18.5 embryos. Boxes 600 μm in length were placed anterior to the primary fissure, in the presumptive central lobe, and posterior to the secondary fissure. The number of GCPs contained in each box was counted on three sections from the most medial 100 μm of each embryo. Data was obtained from three embryos of each genotype. The area encompassed by the IGL in saggital sections was calculated using MetaMorph software. Three representative sections from the most medial 100 μm were used from mutant and control littermates from three different litters.

Results

Shh signaling is dynamic and spatially patterned along the AP axis during cerebellum development

Previous studies have shown Shh and components of the Shh pathway to be expressed in the developing mouse cerebellum around birth (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). However, the temporal onset and progression of Shh signaling and Gli expression in mouse has not been fully documented. In order to determine

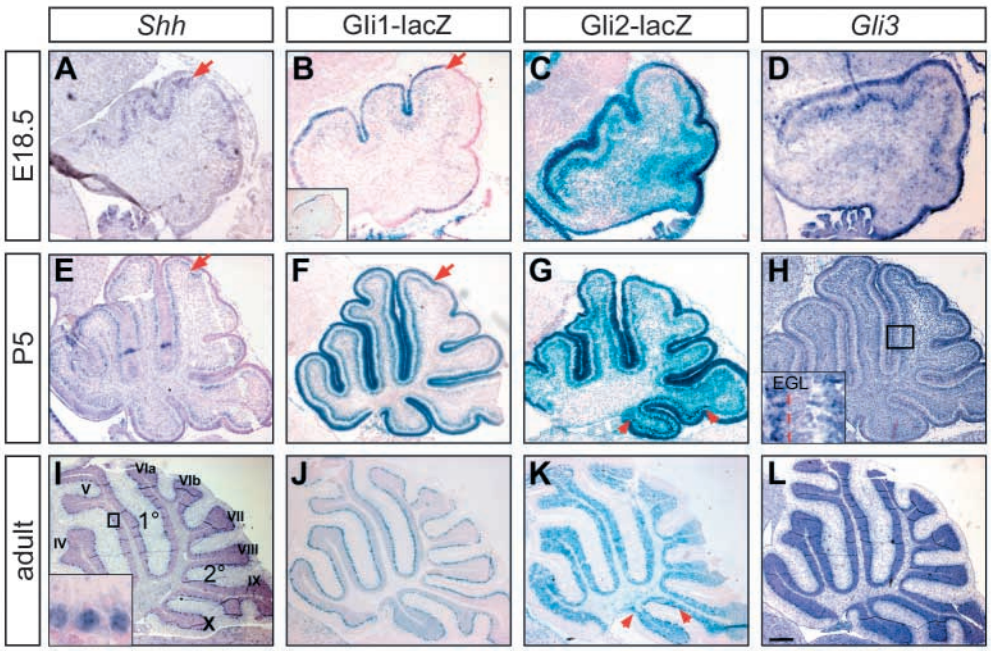

Fig. 1. *Shh* and the downstream factor genes *Gli*, *Gli2* and *Gli3* are expressed in the developing cerebellum. RNA in situ hybridization shows expression of *Shh* in the PCL at E18.5 (A), P5 (E) and adult (P28) (I). *Shh* expression is strongest in anterior regions during early stages (arrows), as well as posterior to the secondary fissure and appears homogenous in the PCL in the adult. Inset in I is a high magnification image of Purkinje cell layer indicated by box. β-Gal activity from the *Gli* locus reveals positive Shh signaling in areas corresponding to *Shh* expression. At E18.5, Gli-lacZ is strongest anteriorly (arrow) and expression is also observed posterior to the secondary fissure (B). In lateral sections, strong Gli-lacZ is observed only in the anterior cerebellum (inset, B). By P5, the intermediate region expresses Gli-lacZ and expression remains stronger in the EGL and PCL anteriorly and posterior to the secondary fissure (F). In the adult, PCL expression is homogenous although IGL expression was higher after 24 hours incubation anterior to VIa and posterior to the secondary fissure (J). Gli2-lacZ was expressed in the EGL and deeper layers along the AP axis at E18.5 (C) and P5 (G). Staining appears weaker between anterior and posterior regions due to a thinner EGL at E18.5. Gli2-lacZ in the adult is present in the IGL and PCL equally along the AP axis (K). At E18.5, *Gli3* expression is detected uniformly in the EGL and deeper layers (D). By P5, *Gli3* remains homogeneous along the AP axis, and stronger expression is observed in the outer EGL (H, and inset). In the adult, *Gli3* expression remains broad. Anterior is to the left. Scale bar: 125 μm in A,B,C,D; 250 μm in E,F,G,H; 500 μm in I,J,K,L.

when and where cells respond to Shh signaling in the developing cerebellum, we used Gli1-lacZ expression as a readout of positive Shh signaling. We utilized mice expressing *lacZ* from the *Gli2* locus to examine *Gli2* expression, and performed RNA in situ hybridization to analyze *Gli3*.

Strikingly, Gli1-lacZ expression was found to be spatially restricted along the AP axis during foliation of the cerebellum. Gli1-lacZ was first detected at E18.5 in the EGL and some deeper cells in a restricted pattern in the anterior region of the medial cerebellum and in the region where the most posterior fissure begins to form (Fig. 1B). Gli1-lacZ expression was also restricted to the anterior region of the EGL in lateral sections (Fig. 1B, inset). At P5, Gli1-lacZ expression was detected throughout the AP axis in the EGL and deeper layers, although expression in the central lobes (VIb to IX) was weaker (Fig. 1F). Interestingly, as fissures formed in the central lobe to give rise to lobes VI-VIII, Gli1-lacZ was detected in the central region. Since the level of *lacZ* expression is inversely proportional to the length of time required to detect β-galactosidase (β-gal) activity, we compared X-gal staining after short (4-6 hours) and long (overnight) incubations (Fig. 1 and data not shown). The highest levels of β-gal activity were detected in the anterior and most posterior lobes by 4 hours of incubation (data not shown). Gli1-lacZ does not appear to be differentially expressed within each lobe, as any subtle differences probably reflect the variable thickness of the EGL during early lobe formation. By P28, when foliation is complete and the EGL has been depleted, expression of *Gli1* was strongest in the Purkinje cell layer (PCL), which also contains Bergmann glia, and this expression was homogeneous along the AP axis (Fig. 1J). Weak expression was also detected in the IGL in lobes III to VIa, in the posterior half of lobe IX, and in lobe X, but only after staining for 24 hours.

The expression of *Gli1* could be due to a similar restricted expression domain of Shh or because the response to Shh is spatially restricted. To address this we analyzed *Shh* mRNA expression and found it to correlate with the pattern of *Gli1* expression during cerebellum development. In midsagittal sections through the cerebellar vermis, *Shh* mRNA was first detected at E18.5 in a deep layer, and was highest in the developing rostral three lobes (III-VIa) and lobe IX, similar to Gli1-lacZ (Fig. 1A). At P5, *Shh* expression could be discerned in the PCL and although it was detected throughout the AP axis, it was lowest in the central lobes VIb-IX (Fig. 1E). By the adult stage, *Shh* mRNA was detected at uniform levels in all Purkinje cells along the AP axis of the cerebellum, similar to Gli1-lacZ in the PCL (Fig. 1I).

To determine which Gli proteins could be activating *Gli1* transcription, we examined the expression of *Gli2* using mice expressing *lacZ* from the *Gli2* locus, and *Gli3* expression was determined using RNA in-situ hybridization. Gli2-lacZ expression was quite distinct from the pattern of *Shh* and Gli1-lacZ. Cerebellar expression of *Gli2* was detected by E15.5, earlier than the onset of *Gli1* and *Shh* (data not shown). At E15.5 and E18.5, Gli2-lacZ was expressed without spatial restriction in the EGL and deeper layers of the cerebellum (data not shown and Fig. 1C). During later stages and through to the adult, strong Gli2-lacZ expression was detected broadly in the cerebellum (Fig. 1G,K).

RNA in situ hybridization with a *Gli3* antisense cDNA probe was carried out to determine the developmental profile of *Gli3* expression in the cerebellum. In a similar way to *Gli2*, *Gli3*

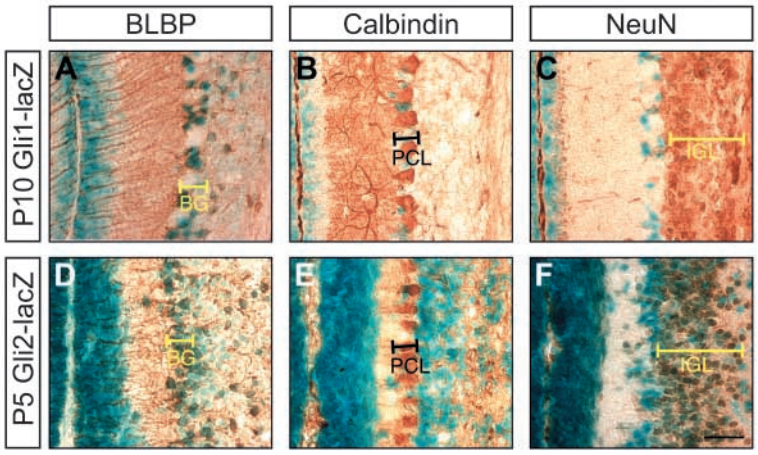

Fig. 2. *Gli* and *Gli2* are expressed in specific cell types in the cerebellum. Expression of *Gli* and *Gli2* co-localize with BLBP, a marker for Bergmann glia (A,D). Purkinje cells, marked with Calbindin, do not express *Gli* and a few express *Gli2* (B,E). *Gli* expression is restricted to the proliferative GCPs. The inner EGL and IGL, marked by NeuN, do not express *Gli* at high levels (C). However, *Gli2* expression is observed throughout the EGL and IGL (F). Bars indicate layers containing Bergmann glia nuclei (BG) in (A,D), Purkinje cell layer (PCL) in (B,E) and IGL (C,F). Scale bar: 50 μm.

was not spatially patterned and was expressed in the EGL and the deeper layers at E18.5 (Fig. 1D). By P5, expression was maintained at similar levels along the AP axis in the EGL and deeper layers (Fig. 1H). Like that of *Gli1*, *Gli3* expression in the EGL was stronger in the outer EGL (Fig. 1H, inset). In the adult, *Gli3* was expressed broadly in most layers and appeared homogenous along the AP axis (Fig. 1L). Thus, *Gli2* and *Gli3* are broadly expressed throughout cerebellum development and do not correlate with the temporally and spatially restricted *Shh* and Gli1-lacZ expression.

Gli1 and Gli2 are expressed in specific cell types of the cerebellum

A primary site of Shh signaling during cerebellum development is clearly the EGL, which is divided into an outer proliferative layer and an inner differentiating layer. Interestingly, Gli1-lacZ expression was strongest in the outer layer of the EGL (Fig. 2A-C). Strong expression of both *Gli1* and *Gli2* was also observed in deeper layers, which could be due to expression in migrating granule cells, Purkinje cells, and/or Bergmann glia that are in close proximity to the Purkinje cells. In order to determine which cell types express Gli1 and Gli2, cryosections from $Gli1^{lz/+}$ and $Gli2^{lz/+}$ P5 and P10 mice were stained with X-gal and subjected to immunohistochemical labeling with cell-type-specific antibodies (Fig. 2 and data not shown). Antibodies against BLBP, NeuN and Calbindin were used to mark Bergmann glia, differentiated granule neurons and Purkinje cells, respectively.

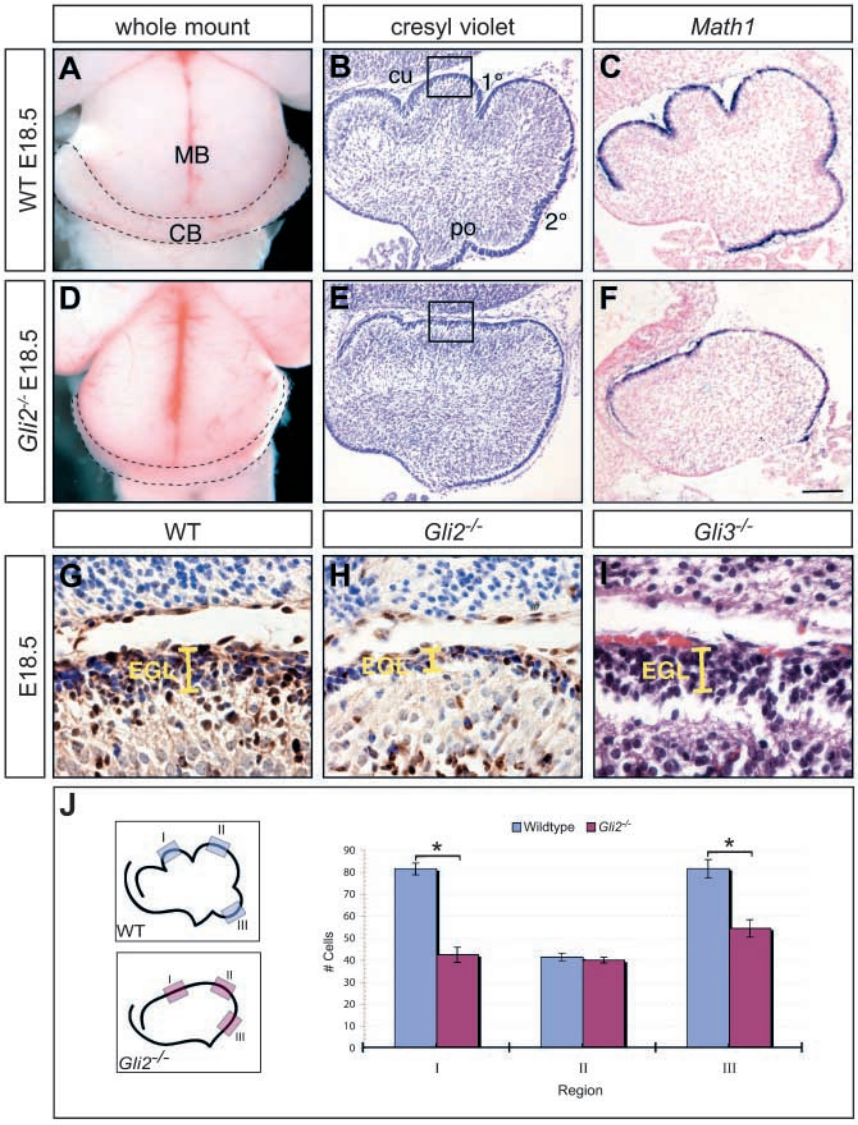

Fig. 3. *Gli2* is required for expansion of the EGL and foliation at birth. Whole-mount analysis of WT and $Gli2^{-/-}$ brains reveals a cerebellar phenotype. The mutant cerebellum (D) is smaller than that of the WT (A). Cresyl Violet staining of sagittal sections through medial WT (B) and mutant (E) brains shows reduced foliation in the mutant. *Math1* expression indicates the presence of GCPs in the EGL of WT (C) and mutant (F) cerebella. High magnification image of PCNA labeling in the EGL demonstrates that the proliferative layer is thinner in mutants (H) compared with WT (G). Hematoxylin and eosin staining of $Gli3^{-/-}$ brains shows that the EGL thickness is similar to WT (I). Region depicted in G,H is indicated in B,E. Anterior is to the left. EGL cell counts from three regions in WT and *Gli2* mutant cerebellar sections were compared (J). In the mutant, regions I and III contain significantly fewer GCPs than WT at E18.5. Error bars indicate the s.d. Student's *t*-test was performed and showed a significant difference between WT and mutant in regions I and III (*P<0.0001). Scale bar: 200 μm in B,C,E,F; 80 μm in G,H,I.

Expression of Gli1-lacZ and Gli2-lacZ in Bergmann glia was demonstrated by coexpression of *lacZ* with BLBP at P5 and P10 (Fig. 2A,D and data not shown). In the adult, when Gli1 expression is homogeneous along the AP axis, Gli1-lacZ was maintained at high levels only in Bergmann glia (data not shown). *Gli2* expression in the adult was at similar levels in Bergmann glia and other cell types. Antibody labeling to detect Calbindin at P10 demonstrated that Gli1 is not expressed in the Purkinje cells, suggesting that positive Shh signaling is non-autonomous in the cerebellum (Fig. 2B). Consistent with this, most Calbindin-positive cells also did not express Gli2 (Fig. 2E), except for a few Purkinje cells, primarily in the posterior lobes (Fig. 1G, between arrowheads, 1K, lobe X). Antibody staining for NeuN, which marks differentiating granule cells, confirmed that Gli1 expression is highest in the outer EGL. The cells in the innermost layer of the EGL and the differentiated GCs in the IGL did not express high levels of Gli1-lacZ (Fig. 2C). By contrast, Gli2 was expressed at similar levels in the inner and outer EGL and also in the IGL (Fig. 2F). Thus, Gli1 is downregulated soon after granule cells begin to differentiate, whereas Gli2 is not.

Gli2 is required to generate a multi-layered EGL and to promote cerebellum foliation at E18.5

To determine whether the Gli proteins are required for granule cell proliferation, we analyzed *Gli2* and *Gli3* mutants, since *Gli1* mutants have a normal cerebellum (Park et al., 2000) (see Fig. 8A). The thickness of the EGL appeared normal in *Gli3* mutants (Fig. 3I), however we cannot conclusively address Gli3 function in GCP expansion due to early patterning defects in rhombomere 1 (S.B. and A.L.J., unpublished). A recent study suggested that the E18.5 *Gli2* mutant cerebellum has abnormal foliation that is more pronounced posteriorly, but a detailed analysis was not performed (Palma and Ruiz i Altaba, 2004). To further explore positive Shh signaling in GCP proliferation and cerebellar foliation, we analyzed *Gli2* mutant mice at E18.5, since the mutants die at birth. This is shortly after the time when a response to Shh signaling is first detected by analysis of Gli1-lacZ. Although the cerebellum is quite immature at E18.5, whole-mount analysis of brains from E18.5 embryos clearly revealed that the cerebellum was smaller in *Gli2* mutants (n=7) compared with normal littermates (Fig. 3A,D). Sagittal sections further showed an almost complete lack of foliation in medial regions (Fig. 3B,E). Furthermore, in comparison with the normal EGL in the anterior and posterior regions, which contained five to eight cell layers at E18.5 (Fig. 3B,G), the *Gli2* mutant EGL contained only two to four cell layers (Fig. 3E,H). In the developing central lobe and in lateral sections, where foliation initiates after birth, the thickness of the EGL and shape of E18.5 *Gli2* mutant cerebella was similar to that of WT (Fig. 3B,E and data not shown). In order to further address whether the EGL in *Gli2* mutants is primarily affected in the regions that receive a high level of Shh, we quantitated the thickness by counting the number of cells in the EGL in three regions along the AP axis (see Materials and methods and Fig. 3J). In areas where Gli1-lacZ is expressed, indicated schematically by regions I and III in Fig. 3J, the mutant EGL was reduced to 50-60% (P<0.0001) of the WT EGL. However, in the central lobe, region II, where Gli1-lacZ was not detected at E18.5, the WT and mutant EGL had a similar thickness.

We next used RNA in situ hybridization with EGL markers, *Math1* (*Atoh1* – Mouse Genome Informatics) and *Pax6*, to confirm that the cells remaining in the *Gli2* mutant EGL were in fact GCPs (Fig. 3F and data not shown). In addition, antibody staining for PCNA, a marker for proliferating cells, was performed to verify that the cells in the mutant EGL were indeed proliferating (Fig. 3G,H). Importantly, at E16.5, the EGL and size of the cerebellum in *Gli2* mutants were indistinguishable from those of normal embryos on sections (data not shown), consistent with the appearance of a phenotype in concert with the onset of Shh signaling in GCPs around E17.5.

In order to address whether cell types other than GCPs were altered in *Gli2* mutants, since one in vitro study indicated Shh induces differentiation of Bergmann glia (Dahmane and Ruiz-i-Altaba, 1999), immunohistochemistry was performed on paraffin sections from E18.5 WT and *Gli2* mutant cerebella. Antibody marker analysis using Calbindin (Fig. 4A,E) and BLBP (Fig. 4B,F) demonstrated that both Purkinje cells and Bergmann glia were present in *Gli2* mutants. At this stage, Purkinje cells are not laminated in a single cell layer. The Purkinje cells in *Gli2* mutants appeared more clustered than in WT cerebella; however, this is probably due to the decreased surface area of the mutants resulting from the decreased GCP pool and lack of foliation. Therefore, the cerebellum phenotype in *Gli2* mutants at E18.5 is probably due to a lack of Shh signaling to the EGL.

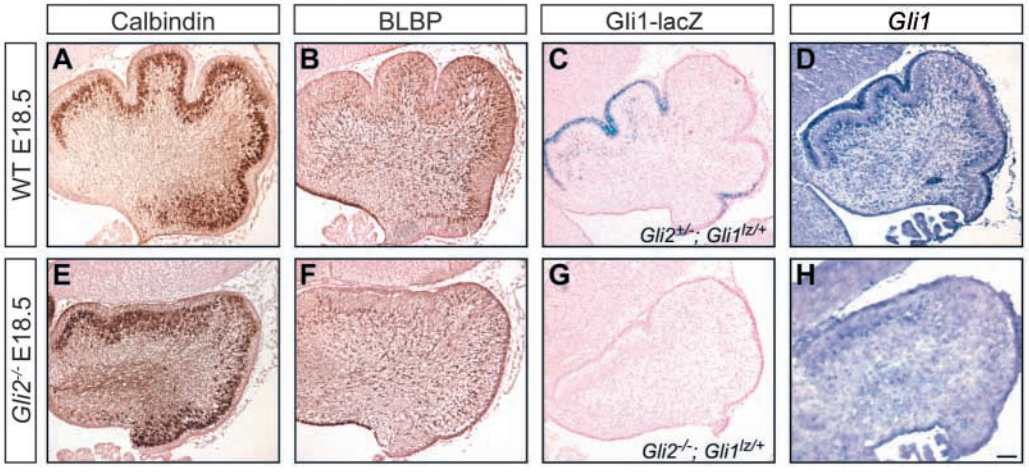

Fig. 4. The *Gli2*$^{-/-}$ phenotype is specific to the EGL. Antibody marker analysis shows that Purkinje cells marked with Calbindin (A,E) and Bergmann glia marked with BLBP (B,F) are present and their general cellular organization appears normal in *Gli2*$^{-/-}$ embryos at E18.5. Gli-lacZ is not detectable in *Gli2*$^{-/-}$ (compare C and G). However, *Gli* mRNA is detectable by RNA in-situ hybridization in *Gli2*$^{-/-}$ embryos (H), but its levels are much weaker than in WT (D). Anterior is to the left. Scale bar: 100 μm.

Finally, Shh-positive signaling was assayed in *Gli2* mutants by analyzing Gli1-lacZ activity and *Gli1* mRNA expression in E18.5 $Gli2^{-/-}$ embryos. Whereas Gli1-lacZ expression was obvious in the anterior cerebellum of E18.5 WT embryos (Fig. 4C), no Gli1-lacZ could be detected in $Gli1^{lz/+}$; $Gli2^{-/-}$ cerebella (Fig. 4G). In addition, RNA in-situ hybridization analysis of *Gli1* expression in $Gli2^{-/-}$ cerebella showed drastically reduced levels of *Gli1* (Fig. 4H) compared with those of WT (Fig. 4D). Since *Gli1* is a direct transcriptional target of positive Shh signaling, these results demonstrate that *Gli2* is the main activator of Shh-positive signaling in the cerebellum.

Overexpression of Shh in the cerebellum of *Shh-P1* transgenics produces larger lobes and an irregular inner granule layer

To determine whether elevated levels of Shh can increase proliferation of GCPs and induce alterations in cerebellar foliation, we employed a gain-of-function approach utilizing transgenic mice (*Shh-P1*) carrying a 100 kb P1 clone that contains the entire *Shh* coding region and some regulatory sequences (Riccomagno et al., 2002). Gross inspection of cerebella from adult transgenics showed that they were larger than normal, especially in the AP axis, but the basic foliation pattern appeared intact (Fig. 5A,E). Histological analysis confirmed this and revealed a thicker IGL, as well as larger lobes in the vermis and hemispheres compared with WT cerebellum (Fig. 5B,C compared with 5F,G). The IGL was thickest in lobes III, IV, V and IX, which correlates with the normal expression pattern of *Shh*. In addition, the IGL surrounding the primary fissure was irregular with distinct bulges. Measurements of the area occupied by the IGL in *Shh-P1* mutants (see Materials and methods), showed a 30% overall increase compared with those of WT.

The *Shh-P1* transgene is known to be missing some negative regulatory elements, since misexpression is observed in the inner ear (Riccomagno et al., 2002). To determine whether the transgene results in ectopic and/or overexpression of *Shh* in the cerebellum, we utilized $Gli1^{lz/+}$ mice as a functional readout of Shh signaling. X-gal staining of sagittal sections from P0 and P5 $Gli1^{lz/+}$; *Shh-P1* double transgenic brains revealed an increased intensity of X-gal staining in the mutant cerebellum (Fig. 5D,H). Importantly, the labeling appeared in an anteriorly restricted pattern identical to the pattern in WT mice (Fig. 5D,H and data not shown). Therefore, the transgene drives overexpression in regions of endogenous *Shh* expression rather than ectopic expression.

The cerebellum begins to expand by P8 in *Shh-P1* mice

In order to determine when and how the *Shh-P1* phenotype arises, transgenic brains were collected and sectioned from early postnatal to adult stages. At P2 (data not shown) and P5 (Fig. 6A,E), the overall morphology of the *Shh-P1* cerebellum (n=5) was similar to that of normal (n=5) littermates (Fig. 6A). If Shh normally elicits a proliferative response in GCPs in the EGL, then an elevated level of Shh signaling might result in thickening of this layer. However, analysis of sections at high magnification did not reveal an obvious difference in the thickness of the EGL in P5 mutant cerebella compared with WT. Consistent with this, labeling with PCNA showed that at P5 in *Shh-P1* transgenics and WT littermates the thickness of the outer EGL was similar (Fig. 7A,E). At P8, in contrast to P5, the IGL was thicker in *Shh-P1* brains than in WTs (n=4) (Fig. 6B,F). As with P5 *Shh-P1* cerebella, however, the thickness of the EGL appeared normal. By P14 (n=3) (Fig. 6C,G) and at P28 (n=4) (Fig. 6D,H), the phenotype of transgenic mice was similar to that of adult mice (n=5). The IGL was thickest and irregular in anterior regions of the P28 cerebellum. These results show that the cerebellar phenotype is first obvious when a compact IGL becomes apparent.

The IGL of P28 cerebella appeared more irregular than at P14, suggesting the phenotype becomes more pronounced after P14. One possibility was that depletion of the EGL is delayed, and GCPs continue to proliferate longer in *Shh-P1* transgenics. In normal mice, most GCPs have differentiated and progressed into the IGL by ~P14. By contrast, in *Shh-P1* mutants the EGL

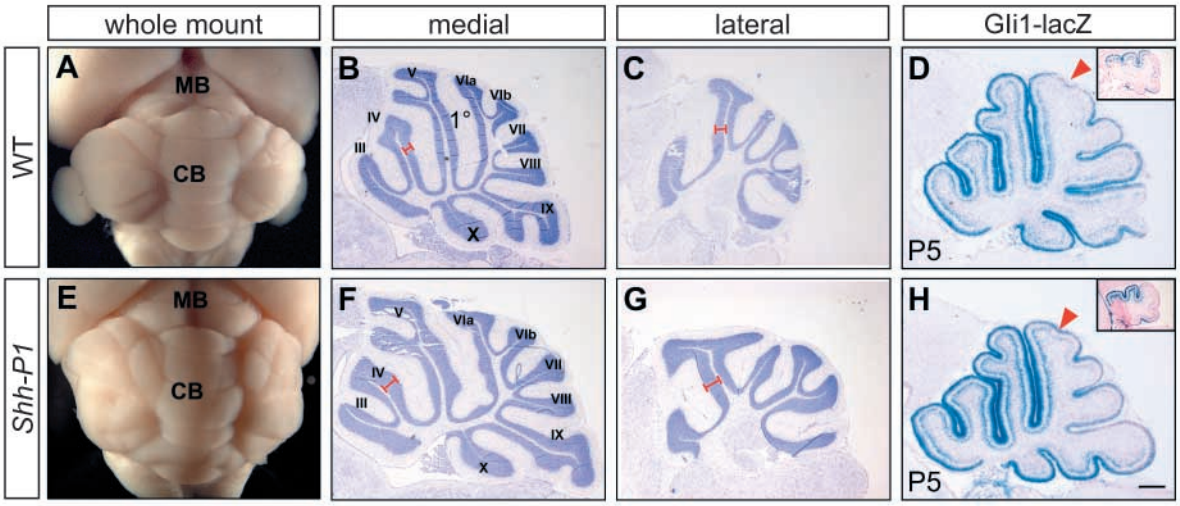

Fig. 5. *Shh-P1* mutants have a larger but well patterned cerebellum. Dorsal views of adult mutant brains (E) show the transgenic cerebellum is much larger than the WT (A). Cresyl Violet staining of paraffin sections of adult mutant cerebella shows enlargement of the cerebellum and a thicker IGL (indicated by bar) in medial (F) and lateral (G) sections, compared with WT (B,C). In the vermis, the phenotype is more severe in lobes III, IV, IV and IX and around the primary fissure. At P0 (inset, D,H) and P5, Gli-lacZ expression is maintained in its normal pattern in *Shh-P1* mutants (H), although at higher levels than in WT (D). In sections, anterior is to the left. Scale bar: 400 μm in B,C,F,G; 250 μm in D,H.

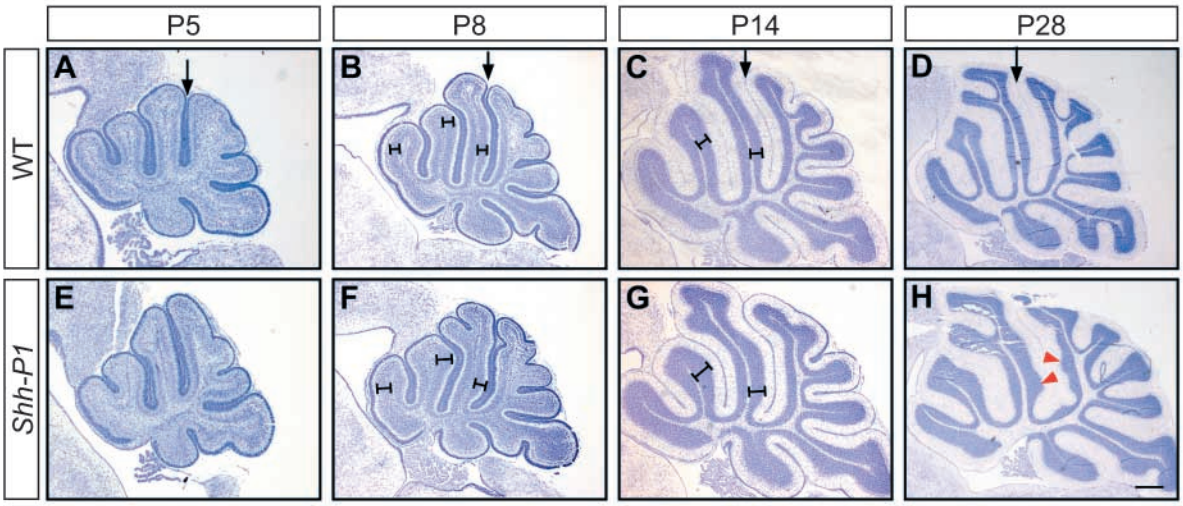

Fig. 6. The cerebellum in *Shh-P1* mice overgrows after P5. Midsagittal sections of WT and mutant brains at P5 appear morphologically similar (A,E). By P8, the mutant IGL (indicated by bars) begins to appear thicker (B,F). At P14, the mutant IGL is noticeably thicker (G) compared with WT (C). The phenotype is most apparent at P28 when the IGL is also irregularly shaped (arrowheads in H), particularly around the primary fissure (arrows). [Note: Fig. 6D,H are duplicated from Fig. 5B,F and placed here for comparison to other stages.] Scale bar: 350 μm in A,E; 500 μm in B,C,F,G; 400 μm in D,H.

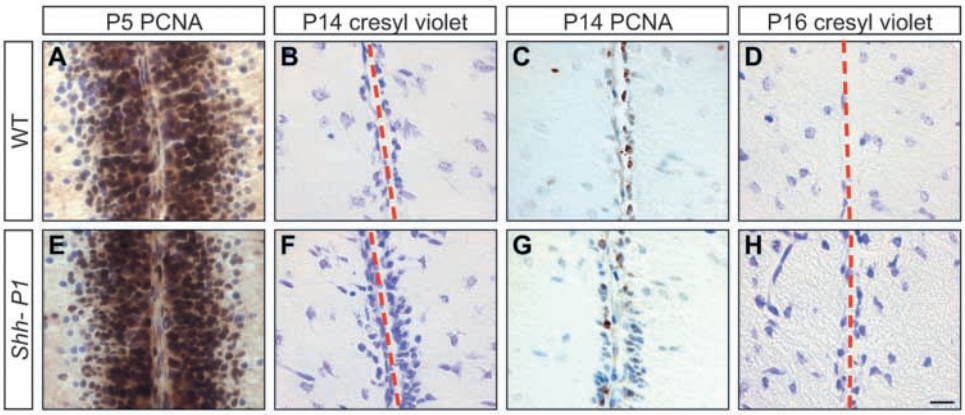

Fig. 7. The *Shh-P1* EGL is not thicker at early stages, but persists longer than in WT cerebella. Antibody staining for PCNA at P5, a marker for proliferating cells, appears similar in WT (A) and mutant (E) EGL. Cresyl Violet staining of P14 sections shows the EGL is one cell layer thick in WT (B), and three to four cell layers thick in mutants (F). PCNA labeling shows the presence of proliferating cells in the EGL at P14 in both WT and mutant (C,G). By P16, the EGL has been depleted in WT (D), but one cell layer is still present in the mutant (H). Red dashed line indicates division between two lobes. Scale bar: 50 μm.

contained three to four layers of cells at P14 (Fig. 7F), compared with a single cell layer in normal mice (Fig. 7B). Furthermore, the EGL in mutants persisted until P16 (Fig. 7H) when the EGL in normal littermates was gone (Fig. 7D). We performed antibody labeling with PCNA and found that both the WT and mutant EGL contained proliferating cells at P14 (Fig. 7C,G) and the EGL cells in the mutant continued to proliferate at P16 (data not shown). Finally, Bergmann glia appear morphologically normal and at normal density (data not shown).

The phenotype of *Shh-P1* transgenics is sensitive to the number of copies of endogenous *Shh*, but not *Gli1*

Since *Gli1* transcription is upregulated in *Shh-P1* transgenics, we were interested in determining whether removal of this activator of Hh targets could rescue the *Shh-P1* phenotype. To address this, *Shh-P1; Gli1*$^{-/-}$ mice were produced and compared with *Gli1* mutants, which have normal cerebella, and *Shh-P1* single mutant mice. Based on whole-mount analysis (n=5, data not shown) and histological sectioning (n=3; compare Fig. 8A-C), *Shh-P1; Gli1*$^{-/-}$ mice appeared similar to *Shh-P1* mice, showing that removal of *Gli1* does not rescue the mutant phenotype. It is possible that the Gli2 activator is sufficient to mediate positive Shh signaling in the absence of Gli1, but this could not be addressed since *Gli2* mutant mice die at birth.

We next tested whether the cerebellum phenotype was sensitive to the number of copies of the endogenous *Shh* gene by analyzing the cerebella of mice carrying the transgene and heterozygous for a *Shh* null mutation (*Shh-P1; Shh*$^{+/-}$ n=3). Of significance, removing one allele of *Shh* (Chiang et al., 1996) partially rescued the *Shh-P1* phenotype (compare Fig. 8B,D). The partial rescue was variable between animals: two of four animals displayed rescue at the level shown in Fig. 8D, whereas the other two *Shh-P1; Shh*$^{+/-}$ mice only showed a slightly smaller cerebellum than *Shh-P1* mice. Histological analysis of *Shh-P1; Shh*$^{+/-}$ cerebella showed that the IGL was not as thick or as irregular as in *Shh-P1* transgenics, and the overall size was reduced.

Discussion

Shh expression and signaling is spatially patterned

It is of interest to determine how the pattern of fissures arises

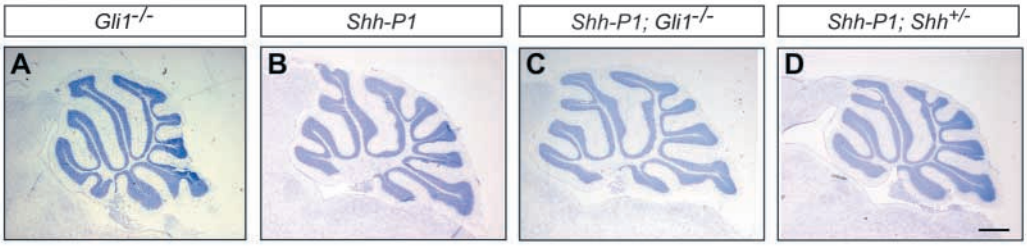

Fig. 8. Lowering *Shh* but not *Gli* levels partially rescues the *Shh-P1* phenotype. Sagittal sections were analyzed for morphology. Removal of the downstream activator Gli does not affect cerebellar size (A) and does not show rescue of the mutant phenotype in double mutants (C), demonstrating that Gli is not the major activator of Shh signaling in the *Shh-P1* cerebellum. When one allele of endogenous *Shh* was removed, a partial rescue of the *Shh-P1* phenotype was observed (D). Although the cerebellum of *Shh-P1; Shh*$^{+/-}$ was larger than the *Gli*$^{-/-}$ (A), the IGL was not as thick or as irregular as the *Shh-P1* IGL (B). Anterior is to the left. Scale bar: 320 μm.

during cerebellum development in order to gain insight into the relationship between development and function of particular lobes. Our results demonstrate spatially patterned expression of *Shh* and response to Shh signaling in the developing cerebellum around birth. This raises the possibility that another mitogen is responsible for inducing GCP proliferation early in the central lobe of the cerebellum at birth. The positive response to Shh signaling (Gli1-lacZ expression) in the developing anterior vermis and lobe IX was observed in two cell types, the proliferating GCPs in the outer EGL and the Bergmann glia in the PCL. The division between high-level and low-level Gli1-lacZ expression observed between lobes VIa and VIb correlates with the expression borders of other genes that are anteriorly or posteriorly restricted in the cerebellum, such as *En-2*, *Fgf-1*, and *Receptor protein tyrosine phosphatase ρ* (McAndrew et al., 1998; Millen et al., 1995). The same border is also identified in *Meandertail* and *Leaner* mouse mutants, in which the anterior lobes are specifically affected (Herrup and Wilczynski, 1982; Napieralski and Eisenman, 1993; Ross et al., 1990). The spatial pattern of Shh signaling during cerebellum development could either simply reflect the intrinsic patterning of the cerebellum along the AP axis, or reflect a direct role of Shh in the process. Of possible relevance, at E18.5 expression of Gli1-lacZ and *Shh* initiate at the same time the four principal fissures divide the cerebellum into five cardinal lobes. In concert with the detection in Gli1-lacZ and *Shh* in the central region of the cerebellum at P5, fissures form to divide the central lobe into lobes VI-VIII. Lobes V and IX undergo the greatest increase in length, and are in areas that endure the longest temporal response to Shh signaling. We propose that Shh signaling is not required for a basal level of proliferation in the EGL, but is required to enhance the level of proliferation required for lobe growth. In accordance with this idea, [^{3}H]thymidine labeling experiments to birthdate GCs in the rat cerebellum demonstrated that the GCs in the central lobes are the latest born, after those that comprise the anterior and posterior regions (Altman and Bayer, 1997). We found that, by overexpressing Shh in the normal domain, all the lobes and IGL enlarge, with greatest effects in the anterior lobes. Although it is not clear whether Shh regulates the position of fissures, based on our studies we suggest that Shh regulates the size and shape of the lobes by influencing the degree of GCP proliferation.

The responsiveness of granule cells to Shh is regulated

The observation that Gli1-lacZ is expressed at highest levels in the outer EGL and Bergmann glia, and at low levels in the inner EGL and IGL, demonstrates that the response of cells to Shh signaling is precisely regulated. This response does not correlate with their proximity to the source of Shh (Purkinje cells), since Bergmann glia express high levels of Gli1-lacZ and the immediately adjacent IGL cells express low levels. Furthermore, *Gli2* and *Gli3* are broadly expressed in the cerebellum, and therefore the response of cells to positive Shh signaling is not regulated at the level of availability of the Gli activators. Previous studies support a role for the extracellular matrix (ECM) in regulating the proliferative response to Shh (reviewed by Wechsler-Reya, 2001). GCPs in the outer EGL are in contact with laminin, whereas differentiating granule cells in the inner EGL and IGL are in contact with vitronectin. Furthermore, GCPs proliferate extensively in the presence of Shh when cultured on laminin, but not vitronectin. Although it remains unknown how the ECM influences Shh signaling, the spatial restriction of particular ECM molecules provides at least one mechanism for the specific activation of Shh signaling we observed in GCPs in the outer EGL.

A mechanism for Shh to elicit a proliferative response in GCPs is by inducing the proto-oncogene *Nmyc*, which has been shown to be a direct target of Shh (Kenney et al., 2003). Similar to Gli1-lacZ, *Nmyc* is expressed in the proliferative outer EGL. In accord with the mitogenic role of Shh in the cerebellum, conditional mutant mice in which *Nmyc* is deleted in the neuroepithelium display severe cerebellar hypoplasia due to a reduced population of neuronal progenitors (Knoepfler et al., 2002). However, these mutants lack *Nmyc* during early formation of the cerebellar anlage; therefore, the GCP pool may be compromised before Shh activity is required for later expansion of the EGL.

Overexpression of *Shh* causes expansion of the folia and inner granule layer

Increased levels of *Shh* in the cerebella of *Shh-P1* transgenics result in overall enlargement of the cerebellum. In addition, there is a greater thickening of the IGL and distinct bulges in the anterior vermis, where Shh levels are highest and maintained over the longest time period during development. The basic foliation pattern, however, is intact. During development, the morphology of the mutant cerebellum appears normal until P8, the stage at which the IGL is first densely populated and becomes tightly compact.

Interestingly, the *Shh-P1* cerebellum resembles an exaggerated version of the normal cerebellum. Specifically, the

primary and invariant fissures are elongated and more distinct in the mutant, whereas the variable fissures seen in lobes V and VI of some WT mice are exaggerated and consistently seen in all *Shh-P1* transgenics. By the adult stage in *Shh-P1* transgenics the IGL has abnormal bulges surrounding the primary fissure, probably reflecting a greater increase in granule cells in areas of highest levels of Shh signaling. The primary fissure increases the most in length, and also forms in a region that responds to high levels of Shh signaling over the longest period of time. In *Shh-P1* mutants, this region is subjected to an increased level of Shh due to the transgene for a longer period of time than the central region. Although PCNA staining and the outer EGL appear normal during early cerebellar development in *Shh-P1* transgenics, the increased number of granule cells that make up the thicker IGL probably results from generating an increased number of GCPs. Therefore, the phenotype of *Shh-P1* transgenics suggests that the level of Shh signaling influences the differential growth of each lobe.

The division of the EGL into a proliferative and non-proliferative layer raises the issue of how the GCPs move from one layer to the other. Movement could be coupled to differentiation, or alternatively, proliferation in the outer EGL could force cells into the inner EGL if the layer could not expand in length indefinitely. In *Shh-P1* transgenics, the outer EGL thickness does not increase, although the overall length of the EGL is expanded due to increased lobe size. This suggests that GCPs differentiate normally even when exposed to excess Shh. The granule cells in *Shh-P1* transgenics move properly into the inner EGL and subsequently into the IGL, although perhaps at a faster rate since the overall size of the IGL is increased by P8. This indicates that the mechanism by which cells exit the cell cycle is intact in these mutants, preventing the accumulation of cells in the EGL. We did, however, find that the EGL persists in *Shh-P1* transgenics for at least two more days than usual, similar to mouse mutants lacking the cell cycle inhibitor p27/Kip1 (Miyazawa et al., 2000).

Although *Gli1* expression is increased in transgenics, removal of *Gli1* was not sufficient to rescue the phenotype. This is consistent with a previous study showing that removal of *Gli1* in a mouse model of medulloblastoma in which Shh is overexpressed does not lower tumor incidence (Weiner et al., 2002). Furthermore, *Gli2*, and not *Gli1*, is required to mediate positive Shh signaling in the embryo (Bai et al., 2002).

Gli2 is the major activator downstream of *Shh* required in the cerebellum

Our analysis of E18.5 embryos lacking *Gli2* demonstrates a requirement for *Gli2* in the positive response to Shh signaling in GCPs. First, *Gli2* mutants display a reduction in EGL thickness in the regions in which *Shh* is expressed and diminished foliation at E18.5, shortly after the onset of Gli1-lacZ expression, which marks the positive response to Shh signaling. Second, the *Gli2* mutant phenotype seems to be specific to the EGL, as other cell types such as Bergmann glia and Purkinje cells appear normal. Finally, Gli1-lacZ expression is not detected in $Gli2^{-/-}; Gli1^{lz/+}$ embryos, demonstrating that Gli2 is the major activator of Shh signaling in the cerebellum. *Gli3* could play a role only as a weak activator, since *Gli1* expression is very weak in *Gli2* mutants. In support of this, the EGL in *Gli3* mutants is not thinner than normal. The presence of an EGL in *Gli2* mutants, although reduced, suggests that positive Shh signaling is not required for a basal level of proliferation, but induces a heightened level of proliferation. Due to the perinatal lethality of mutations in *Gli2*, a conditional knockout is required to determine the role for *Gli2* in postnatal cerebellum development.

In summary, our studies highlight a mechanism for Shh signaling in the cerebellum that primarily modifies Gli2 into an activator to induce GCP proliferation. Using gene expression analysis and a gain-of-function study, we also demonstrate that the positive response to Shh signaling in the cerebellum does not occur homogeneously along the AP axis. Both the pattern of expression of Gli1-lacZ in the vermis and the phenotype of *Shh-P1* mutants correlate with a compartment border observed by other gene expression patterns and mutations affecting the cerebellum. Furthermore, there is a direct correlation between the temporal onset of fissure formation in different cerebellar regions and the timing of elevated levels of Shh signaling in particular areas. Thus, regulating the level and spatial pattern of Shh may have provided a means during evolution to produce a more complex foliation pattern in higher mammals.

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