Primary Cilia are required for cerebellar development and Shh-dependent expansion of progenitor pool

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Abstract

Cerebellar granule cell precursors (GCPs), which give rise to the most abundant neuronal type in the mammalian brain, arise from a restricted pool of primary progenitors in the rhombic lip (RL). Sonic hedgehog (Shh) secreted by developing Purkinje cells is essential for the expansion of GCPs and for cerebellar morphogenesis. Recent studies have shown that the primary cilium concentrates components of Shh signaling and that this structure is required for Shh signaling. GCPs have a primary cilium on their surface (Del Cerro and Snider, 1972). Here, we show that 1) this cilium can be conditionally ablated by crossing *Kif3a$^{fl/-}$* mice with *hGFAP-Cre* mice, 2) removal of *Kif3a* from GCPs disrupts cerebellar development, and 3) these defects are due to a drastic reduction in Shh-dependent expansion of GCPs. A similar phenotype is observed when Smoothened (Smo), an essential transducer of Shh signaling, is removed from the same population of GCPs. Interestingly, *Kif3a-Smo* double conditional mutants show that *Kif3a* is epistatic to *Smo*. This work shows that *Kif3a* is essential for Shh-dependent expansion of cerebellar progenitors. Dysfunctional cilia are associated with diverse human disorders including Bardet-Biedl and Joubert syndromes. Cerebellar abnormalities observed in these patients could be explained by defects in Shh-induced GCP expansion.

Keywords

Neurogenesis; Primary cilia; Kif3a; Sonic hedgehog; Cerebellar development; Joubert syndrome

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Introduction

The most abundant neurons in the cerebellum, and in the entire brain, are granule neurons. Granule cell precursors (GCPs) arise from a restricted germinal center that develops in the anterior margins of the 4th ventricle known as the rhombic lip (RL) (Altman and Bayer, 1997). In the mouse, the GCPs leave the RL at around embryonic day (E) 13 and migrate rostrally over the dorsal surface of the cerebellar anlage to form the external granule cell layer (EGL). During the first two weeks after birth, cells in the EGL undergo extensive proliferation to produce the pool of GCPs required to generate the large number of granule neurons. Developing GCPs then exit the cell cycle, and migrate internally past the Purkinje cells to form the inner granule cell layer (IGL) (Sotelo, 2004).

Sonic hedgehog (Shh) is a secreted morphogen that plays essential roles during brain development. Binding of Shh to its receptor Patched1 (Ptc1) relieves inhibition of the signaling pathway activated by the transmembrane protein Smoothened (Smo), resulting in the activation of transcriptional targets by members of the Gli family of transcription factors (Ingham, 1998). In the spinal cord, Shh specifies a subpopulation of ventral cell types in a concentration-dependent manner (Chiang et al., 1996; Briscoe et al., 1999). In the limb, Shh is essential for proper morphogenesis and digit identity (Riddle et al., 1993; Litingtung et al., 2002). In the cerebellum, Shh is expressed in Purkinje cells and regulates the proliferation of GCPs (Dahmane and Ruiz i Altaba, 1999; Wechsler-Reya and Scott, 1999; Wallace, 1999). The cellular and molecular mechanisms through which Shh signaling regulates GCP proliferation are still under investigation. However, a number of recent studies suggested that one direct target of Shh signaling is the proto-oncogene N-myc1, which mediates GCP proliferation (Kenney et al., 2003; Knoepfler et al., 2002) via cyclin D1 and cyclin D2 (Kenney and Rowitch, 2000; Ciemerych et al., 2002).

The primary cilium is an antenna-like specialized structure that projects from the surface of many eukaryotic cells (Singla and Reiter, 2006). It has an axoneme with a 9+0 microtubule arrangement and is assembled and maintained by intraflagellar transport (IFT) (Rosenbaum and Witman, 2002). IFT is a specialized intracellular trafficking process that moves multimeric protein complexes bidirectionally along the ciliary axoneme. The anterograde and retrograde movements of proteins are mediated by kinesin-II and cytoplasmic dynein complex, respectively. Kinesin-II is composed of two different motor subunits (KIF3A and KIF3B) and one non-motor accessory subunit (KAP3). Genetic and localization experiments have shown that kinesin-II is essential for the construction and maintenance of cilia (for review see Marszalek and Goldstein, 2000). In the central nervous system (CNS), primary cilia are present in cells in the ventricular zone (VZ) during embryogenesis (Cohen et al, 1988; Huangfu and Anderson, 2005), in granule cell precursors in the EGL at early post-natal stages (Del Cerro and Snider, 1972), in neural progenitor cells (Alvarez-Buylla et al., 1998; Doetsch et al., 1999) and in neurons in the adult (Fuchs and Schwark, 2004; Whitfield, 2004). Recent studies demonstrated that primary cilia are required for the specification of ventral cell types in the spinal cord in response to Shh (Huangfu et al., 2003; Huangfu and Anderson, 2005). Moreover, essential components of Shh signaling, Smo (Corbit et al., 2005), the Gli transcription factors, Suppressor of fused (Sufu) (Haycraft et al., 2005), and Ptc1 (Rohatgi et al., 2007) have been shown to localize to primary cilia.

However, little is known about the function of this intriguing cellular organelle in other cells in the developing CNS.

Here, we have investigated the function of primary cilia in the development of cerebellar GCPs and its contribution to Shh signaling in these cells. Conditional ablation of *Kif3a* in cells derived from Cre-expressing cells under the human glial fibrillary acidic protein (hGFAP) promoter resulted in GCPs without primary cilia. In these animals, GCPs are specified, but a severe defect in late embryonic and early postnatal expansion of GCPs results in atrophied cerebella. We show that the primary cilium is required for Shh-induced GCP proliferation via induction of *Gli1* and *cyclin D1* gene expression. Therefore, our results suggest that the primary cilium in GCPs is required for their Shh-induced expansion and cerebellar development.

Materials and Methods

Transgenic Mice

All animal care was in accordance with the guidelines of the National Institutes of Health, the University of California and European law. All the mice used in this study have been described previously: the *hGFAP::Cre* mouse line containing 2.2 kb of the *hGFAP* promoter linked to the coding region of Cre recombinase (Zhuo et al., 2001), *Kif3a^{fl}* mice containing loxP sites flanking exon 2 of the *Kif3a* gene and *kif3a^{-/+}* mice containing the recombined allele (Marszalek et al., 1999), *R26R* mice (Soriano, 1999), *Z/EG* mice (Novak et al., 2000) and *Smo^{fl}* mice (Long et al., 2001). Mice used in this study were in *C57Bl6/J* or mixed backgrounds.

Immunostaining

Brains were fixed by immersion (embryonic brains) in 4% paraformaldehyde at 4°C for 16 hours or by perfusion (postnatal brains) in the same fixative and washed overnight at 4°C in PBS and cryoprotected in PBS containing 30% sucrose. Histological sagittal sections were cut at 10 or 12 μm on a cryostat and pre-blocked for ICC for 30 minutes to 1 hour in TBS or PBS with 0,1% Triton X-100 and 10% normal goat or horse serum. Sections were incubated overnight at 4°C with the primary antibodies. The following antibodies were used: rabbit polyclonal (pAb) anti-GFAP (1:200; Sigma), chicken pAb anti-GFP (1:500, Aves Labs inc.), rabbit pAb anti-GABA$_A$ receptor α6 subunit (α6, 1:1000, Chemicon), rabbit pAb anti-γ-tubulin (1:1000, Sigma), rabbit pAb anti-CaBP (1:5000, Swant), rabbit pAb anti-Pax6 (Osumi et al., 1997), mouse monoclonal (mAb) anti-Cre (1:500, Euromedex), mouse mAb anti-acetylated α–tubulin (1:1000, Sigma), rat monoclonal (rAb) anti-BrdU (1:200, Oxford Biotechnologies), goat monoclonal (gAb) anti-β–galactosidase (1:700, Biotrend), and species-specific secondary antibodies (Molecular Probes or Jackson ImmunoResearch). Sections were counterstained with DAPI (10 μg/ml, Sigma), mounted in Fluoromount and examined with a fluorescence microscope or a fluorescence confocal microscope (DM IRBE and SP2 Leica).

Isolation of cells enriched in GCPs and Purkinje cells, RNA preparation and quantitative real-time RT-PCR (qRT-PCR)

Cells enriched in GCPs and Purkinje cells were isolated from 2-4-day-old (P2-4) mutant and wild-type pups as described (Hatten, 1985; Baptista et al., 1994; Weschler-Reya and Scott, 1999). Cerebella were digested in solution containing 10 μg/ml trypsin and 0,5 mg/ml DNAse and triturated to obtain a cell suspension. This suspension was centrifuged through 35% and 60% Percoll (Pharmacia), and large and small cell fractions were harvested from the upper phase and the 35%/60% interface, respectively. Glial cells were removed from both fractions by sequential platings on poly-D Lysine coated dishes for 30 minutes at 37°C. To evaluate the purity of Purkinje cells in the large cell fraction, a small aliquot was immediately plated on a dish and immunostained with anti-CaBP antibody. This large cell fraction contains approximately 60% of Purkinje cells (not shown). RNAs from cells were extracted using Quiazol (Qiagen) according to the manufacturer's protocol. Isolated RNA was quantified and analyzed for impurities by ultraviolet spectrometer. Total RNA was reverse transcribed using the SuperScript-II reverse transcriptase (Invitrogen), according to manufacturer's instructions. qRT-PCR was performed with an MX3000P QPCR System (Stratagene). The PCR primers 5'-AAAGCTGACCCCTTTAGCCTA -3' and 5'-TTCGGAGTTTCTTGTGATCTTC -3' were designed to target *Shh* exon 1. The PCR primers 5'- TCGACCTGCAAACCGTAATCC -3' and 5'-TCCTAAAGAAGGGCTCATGGTA -3' targeted the splice junction between *Gli1* exons 4 and 5. The PCR primers 5'- CAGAAGTGCGAAGAGGAGGTC-3' and 5'-TCATCTTAGAGGCCACGAACAT-3' targeted *cyclin D1* exon 2. The housekeeping gene *hprt* was used to normalize the data. To compare the relative amount of *Gli1, cyclinD1* and *Shh* in wt and *hGFAP::Cre; Kif3a$^{fl/-}$* conditional mutant, the Ct values of these genes were divided by the Ct values of *hprt* in the same sample.

Cerebellar slice cultures

Cerebella from wild-type and mutants were removed aseptically, and cut into 300 μm thick parasagittal sections using a tissue chopper. Slices were cultured on Millicel CM culture inserts (Millipore, Bedford, MA) in 6-well plates containing serum-free medium supplemented with 6 mg/ml glucose, 25 mM potassium chloride in the presence or absence of a biologically active N-terminal fragment of Shh (Shh-N; R&D Systems; 3 μg/ml). After 48 hours, BrdU was added to the medium, and slices were cultured for 16 hours. Slices were then fixed with 4% paraformaldehyde, treated with 2 N HCl followed by 0.1 M borate, and stained overnight with anti-BrdU antibody. The next day, slices were washed and stained with alexa 488-anti-rat antibody, counterstained with DAPI and mounted in Fluoromount G. Staining was visualized and quantified using a fluorescent confocal microscope (DM IRBE, Leica, Nussloch, Germany).

Bromodeoxyuridine (BrdU) administrations

BrdU (Sigma, St. Louis, MO) at 50 μg/g body weight (dissolved in 0.9% saline) was injected 1 hour before sacrifice at E14.5, E16.5, E18.5 or P0. BrdU was injected intraperitoneally into the mother or subcutaneously into the young mice postnatally. For each animal analyzed, the total number of BrdU+ cells in the EGL in each of lobe I to IV

and RL region was counted on 3 sagittal sections of comparable mediolateral levels (n=3) using a fluorescent microscope (Zeiss, Germany).

In situ hybridization

Antisense riboprobes were labeled as described previously (Spassky et al., 1998) by *in vitro* transcription of cDNAs encoding mouse *Shh* (gift from A. McMahon) or *Gli1* (gift from A. Ruiz i Altaba). In situ hybridization was done as described (Spassky et al., 1998).

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nickend labeling (TUNEL)

Apoptosis was detected by TUNEL assay with the *In Situ* Cell Death Detection Kit (Roche), according to the manufacturer's instructions.

Electron microscopy (EM)

P0 mice were killed by intracardial perfusion with 2% PFA and 2.5% glutaraldehyde. Brains were postfixed in 2.5% glutaraldehyde overnight at 4°C, cut sagittaly at 300 μm, and processed for EM as described previously (Doetsch et al., 1997). Briefly, sections were postfixed in 2% osmium tetroxide in 0.1M PB for 2h. Semithin sections, 1.5 μm thick, were cut with a diamond knife and stained with 1% toluidine blue. For the identification of primary cilia, 300 successive ultrathin (0.06 μm) sections were cut with a diamond knife, stained with lead citrate, and examined under a Jeol (Peabody, MA) 100CX electron microscope.

Results

The cerebellar granule cell lineage is derived from *hGFAP::Cre* expressing precursors

Recent work has demonstrated that GFAP, an intermediate filament protein previously thought to be present in mature terminally differentiated astroglial cells (Bignami et al., 1972), is also expressed by neural stem cells (Doetsch et al., 1999; Laywell et al., 2000; Garcia et al., 2004). Developmental lineage tracing experiments using the mouse or human GFAP promoter also indicate that a subpopulation of embryonic neural progenitors (Malatesta et al., 2003; Casper and McCarthy, 2006), including precursor cells within the cerebellum (Marino et al., 2000; Zhuo et al., 2001, Kwon et al., 2001), express this intermediate filament protein. We thus tested whether GCPs undergo recombination in mice carrying *hGFAP::Cre*. In *hGFAP::Cre* mice, Cre immunoreactivity was detected at embryonic day 14.5 (E14.5) in proliferating progenitors in the cerebellar ventricular zone and in the RL (Fig. 1A-B), but not in the EGL. To visualize the progeny of these cells, we crossed the *hGFAP::Cre* mice with the *R26R* or *Z/EG* reporter mice (Soriano, 1999; Novak et al., 2000). Similar patterns of expression of both reporters were observed at all developmental stages. In E16.5 *hGFAP::Cre; Z/EG* embryos, GFP^{+} cells were found in the RL, EGL and dispersed in the cerebellum, suggesting that they were derived from Cre[+] progenitors in the RL and ventricular zone, and migrated to the EGL (Fig. 1C). We confirmed that GFP^{+} cells in the EGL were GCPs by immunostaining with GCP marker Pax6 (Lin et al., 2001) (Fig. 1D). At all stages examined, GFP^{+} cells were not immunoreactive for the Purkinje cell marker calbindin D28k[16], indicating that these cells are

generated before the onset of Cre expression in the cerebellar ventricular zone (Sotelo, 2004; Fig. 1E and data not shown). At postnatal day 15 (P15) *hGFAP::Cre; R26R* mice, β-galactosidase activity is restricted to Cre+ Bergmann glia in the Purkinje cell layer (PCL) and to α6+ granule neurons in the IGL (Fig. 1F and data not shown). Taken together, these results showed that *hGFAP::Cre* can efficiently recombine genes in GCPs but not in Purkinje cells.

Loss of *Kif3a* in GCPs severely disrupts cerebellar development

During development, Shh expressed in Purkinje cells induce the proliferation of GCPs (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Work from the ventral spinal cord and limb development suggests that the primary cilia plays essential roles in Shh signal transduction (Huangfu et al., 2003; Huangfu and Anderson, 2005, Liu et al., 2005; Haycraft et al., 2005; May et al., 2005). To test whether primary cilia in GCPs are required for cerebellar development, we generated knockouts of *Kif3a* in GCPs by using Cre/LoxP recombination. *Kif3a* encodes a subunit of kinesin-II, an anterograde IFT motor, which is essential for ciliogenesis (Marszalek et al., 1999). *hGFAP::Cre* transgenic mice were crossed with *Kif3a$^{fl/-}$* and *Kif3a$^{fl/fl}$* mice to produce conditional *Kif3a* mutants: *hGFAP::Cre; Kif3a$^{fl/-}$* and *hGFAP::Cre; Kif3a$^{fl/fl}$* mice. Cre-mediated recombination between the loxP sites results in deletion of exon 2, which introduces a frameshift in the coding region resulting in premature termination of translation. PCR analysis of *hGFAP::Cre; Kif3a$^{fl/-}$* progeny showed that the floxed allele was recombined in purified GCPs, resulting in cells with no functional KIF3A (Fig. 2A). Both *hGFAP::Cre; Kif3a$^{fl/-}$* and *hGFAP::Cre; Kif3a$^{fl/fl}$* mice showed the same phenotype. These mice will be called conditional *Kif3a* mutants throughout the manuscript.

To confirm that primary cilia were ablated in GCPs in these conditional *Kif3a* mutant animals, we performed transmission electron microscopy and immunofluorescence analysis using an anti-acetylated α-tubulin antibody to label microtubules within primary cilia, and an anti-γ-tubulin antibody to visualize the basal body. In wild type cerebellum, primary cilia extended from basal bodies in GCPs in the EGL (Supplementary Fig. 1; Fig. 2B, D). In contrast, conditional *Kif3a* mutants completely lacked primary cilia and only had basal bodies. Nine and ten immunofluorescence fields were sampled to detect primary cilia from wild-type and *Kif3a* conditional mutants, respectively. In wild-type, primary cilia were detected from all 9 fields and 77 out of 107 basal bodies were associated with a primary cilium at E18.5. In contrast, only 2 out of 102 basal bodies were associated with primary cilium in 10 fields from conditional *Kif3a* mutants. However, basal bodies were often observed apposed to the cell membrane as in wild type animals, suggesting that the mechanism of basal body anchorage is still functional in conditional *Kif3a* mutants (Fig. 2C, E; Supplementary Fig. 2). The loss of cilia was specific to the cell lineages expressing *hGFAP::Cre*, since neighboring meningeal cells had cilia associated with the basal body (arrow in Fig. 2C). Consistent with *hGFAP::Cre* expression pattern, Bergmann glia also lacked primary cilia in conditional *Kif3a* mutants (data not shown).

At birth, mutant and control pups were indistinguishable. However by P10, the mutants showed ataxia and tremor. Interestingly, these mice spent excessive time digging and

grooming themselves. The vast majority of the mutant mice died around P25. In the forebrain, the *hGFAP* promoter is known to target *Cre* expression in a subset of radial glial cells from E13.5 (Malatesta et al., 2003). At postnatal stages, hydrocephalus develops in the forebrain of *Kif3a* mutants, probably due to defects in ependymal ciliary beating leading to a thinner cortex. Although the overall size of the forebrain in the mutant mice was not altered, the cerebellum of these animals was extremely small compared to control littermates (28±2% of wild type size, Fig. 3A-E). The ratio of the cerebellum to forebrain was 0.35 in control brain and 0.1 in conditional *Kif3a* mutants. A molecular layer (ML), PCL, and IGL were discernable in the severely atrophic P25 mutant cerebella, but there were significant abnormalities with foliation. Folia III, IV and VI, VII were fused (Fig. 3D-E). In the mutant, the total cerebellar area and cell number was significantly reduced. The ML was thinner and contained fewer cells, the PCL was frequently interrupted and Purkinje cell position was not limited to a single row of cells as in the controls (not shown). Instead, in the mutant animals, Purkinje cells formed a thick layer, which is probably a consequence of the reduction in cerebellar surface and reduced number of folia. Indeed, cerebellar growth and foliation are dependent on the expansion of GCPs (Corrales et al., 2006). Consistently, the most dramatic change in the mutant cerebellum from birth was a great reduction in the number of GCPs in the EGL. The defects were already present at birth and became obvious by P5 (Fig. 3F-I). At this age the EGL of mutant cerebellum was much thinner than that of wild-type cerebellum.

This *GFAP*-driven Cre recombinase is also expressed in Bergmann glia (Fig. 1F and data not shown). Granule neurons migrate to the IGL along Bergmann glia fibers (Yacubova and Komuro, 2003), and Bergmann glia differentiation defects leads to layering defects including severe granule neuron migration defects and abnormal laminar formation (Yue et al., 2005). Although Bergmann glial fibers were slightly deformed and disorganized in conditional *Kif3a* mutants compared with the ordered and linear morphology of wild-type Bergmann glial fibers, they expressed the late marker GFAP and their glial fibers extend to the pial surface suggesting that Bergmann glia differentiation proceeds quite far (Supplementary Fig. 3). Moreover, no migratory defects of granule neurons or abnormal laminar formation were detected in conditional *Kif3a* mutants (Fig. 3), indicating that the phenotype observed is not due to Bergmann glia differentiation defects.

Loss of *Kif3a* results in GCP proliferation defects

The observed reduction of GCPs in conditional *Kif3a* mutants could be due to a decreased proliferation or an increase in the number of cells undergoing apoptosis. To test the latter possibility, we first analyzed P0 cerebellum using transmission EM. 300 ultrathin serial sections from control and mutant EGL were carefully studied and the number of picnotic cells counted. No significant increase in the number of cell death could be detected in the mutant compared to wild-type (less than 1% of cells in both animals, data not shown). To confirm these results, we stained sections from control and mutant E18 and P0 cerebellum for TUNEL and activated caspase 3. The number of TUNEL[+] or activated caspase 3^{+} cells in the cerebellum at these developmental stages was very similar in controls and conditional *Kif3a* mice: 10±4 TUNEL[+] (or activated caspase^{+}) cells per section in controls (n=5) and 8±3 per section in conditional mutants (n=6) (Supplementary Fig. 4 and data not shown).

This suggests that increased cell death cannot account for the severe reduction in the number of granule cells observed in the mutant mice.

To test if primary cilia are required for the early proliferation of GCPs, the number of cells incorporating BrdU was examined at E14.5 and 16.5 in control and conditional *Kif3a* mutant cerebella. BrdU^{+} cells were concentrated in the RL and the EGL and scattered in the inner layers of the cerebellum. Mutant mice showed no significant difference in the distribution or the number of BrdU^{+} cells at these early ages (Fig. 4A-C and data not shown). In contrast, at E18.5, conditional *Kif3a* mutant cerebella exhibited a striking reduction in the number of proliferating GCPs as determined by the extent of BrdU incorporation in the EGL (Fig. 4D-F). We counted the number of BrdU[+] GCPs in five regions along the AP axis of conditional *Kif3a* mutants and control cerebellum (see Materials and Methods and Fig. 4D). In rostral areas indicated schematically by regions I and II in Fig. 4D-E, where *Shh* is first expressed (Corrales et al., 2004; Lewis et al., 2004), the number of BrdU[+]cells was significantly reduced in conditional *Kif3a* mutants. In more posterior regions (III, IV) of the cerebellum and in the RL the number of BrdU^{+} cells was only slightly reduced in conditional *Kif3a* mutant animals (Fig.4F) at this stage. At P0, when Shh is expressed in all rostrocaudal regions of the cerebellum, the number of BrdU[+]cells was significantly decreased in all regions of the EGL (data not shown). These results suggest that loss of *Kif3a* results in a dramatic reduction in the proliferation of GCPs and that it coincides in time and space with the pattern of *Shh* expression.

Loss of *Ftm* results in GCP proliferation defects

To provide further evidence for the defective proliferation of GCPs in the conditional *Kif3a* mutants is due to the loss of cilia, we next analyzed the proliferation of GCPs in another ciliary mutant, *Fantom* (*Ftm*, also called *nephrocystin-8* or *Rpgrip1l*) mutant mice. *Ftm* encodes a basal body protein and mutations in this gene cause ciliary disorders including Joubert and Meckel syndromes. Consistently, mutant mice for this gene show defective ciliogenesis and importantly, cerebellar hypoplasia (Delous et al., 2007; Vierkotten et al., 2007). At E18.5, BrdU proliferation was significantly reduced in rostral regions I and II (Fig. 4G-I). The reduced proliferation of GCPs observed in *Ftm* mutant mice in rostral regions of the cerebellum is consistent with our results from *Kif3a* mutant mice, suggesting that primary cilia play an important role in the proliferation of GCPs.

Loss of *Kif3a* in GCP disrupts the expression of Shh target genes

The above results suggested that loss of *Kif3a* in GCPs could cause defective Shh signaling in these cells. In order to examine how this pathway is affected, we investigated if the patterns and levels of expression of *Shh* and its target gene, G*li1*, were altered using *in situ* hybridization and qRT-PCR. At P2, *Shh* was expressed in a deep layer corresponding to the presumptive PCL (Fig. 5A). This pattern of staining is similar to that described previously (Dahmane and Ruiz I Altaba, 1999; Corrales et al., 2004; Lewis et al., 2004). A similar pattern of expression was detected in the conditional *Kif3a* mutant cerebellum, although its expression seemed more pronounced (Fig. 5B). To quantify the expression of *Shh* mRNA in Purkinje cells at P2-4, qRT-PCR was performed using RNA prepared from a large cell fraction, which is enriched with Purkinje cells, isolated from control and conditional *Kif3a*

mutant cerebella (see Materials and Methods). The level of *Shh* mRNA in a large cell fraction isolated from *Kif3a* mutant cerebellum was increased to 400-450% of that found in wild-type (Fig. 5C), indicating that the lack of cilia in GCPs increases the level of expression of *Shh* although it does not affect the pattern of expression of *Shh* in the developing cerebellum. Although approximately 60% of the large cell fraction is Purkinje cells, it was shown that *Shh* is not expressed by Bergmann glia and is restricted to Purkinje cells (Dahmane and Ruiz i Altaba, 1999; Corrales et al., 2004; Lewis et al., 2004), suggesting that the level of *Shh* mRNA expression in Purkinje cells in *Kif3a* mutant is increased compared to controls. To determine whether Shh signaling was disrupted in conditional *Kif3a* mutants, the expression of *Gli1*, a Shh downstream target gene, was analyzed in P2-4 conditional *Kif3a* mutants and control animals. As previously described, *Gli1* was detected by *in situ* hybridization in the EGL and in some deeper cells in the anterior region of the control cerebellum, corresponding to developing Bergmann glia (Fig. 5D; Corrales et al., 2004). In contrast, very low or no expression of *Gli1* could be detected in the mutant GCPs by *in situ* hybridization or qRT-PCR (Fig. 5E-F). Shh mediates GCP proliferation via cyclin D1, as *cyclin D1* mRNA is also upregulated in response to Shh in GCPs (Kenney and Rowitch, 2000). We therefore compared the expression of *cyclin D1* in the control and mutant GCPs using qRT-PCR. Although *Shh* was upregulated in Purkinje cells of the mutants, the level of *cyclin D1* expression in mutant GCPs was reduced by 50% compared to that found in wild-type GCPs (Fig. 5G). These findings indicate that while *Shh* was expressed in the cerebellum at the right time and place, the downstream genes, *Gli1* and *cyclin D1*, were dramatically down-regulated. These findings suggest that Shh target genes fail to be expressed in GCPs in the absence of primary cilia.

Kif3a is required for Shh-induced proliferation of GCPs

The phenotype, timing of the defect and expression of Shh downstream genes, *Gli1* and *cyclin D1*, in the conditional *Kif3a* mutant mice, suggest that these mice have a defect in Shh induced proliferation in GCPs. To test if primary cilia in GCPs are required for Shh-induced proliferation, we examined the effect of the biologically active N-terminal fragment of Shh (Shh-N) on cerebellar slice cultures (Weschler-Reya and Scott, 1999). Slices of P3 cerebellum from conditional *Kif3a* mutant and control littermates were cultured for 48 hours in the presence or absence of Shh-N. Proliferating cells in these slices were labeled with BrdU and quantified from single optical slices through the EGL using a confocal microscope by counting the percentage of BrdU+ cells per DAPI total cells. EGL boundaries were determined by BrdU and DAPI staining on each section. In control slices from wild type mice, exposure to Shh-N resulted in a 10-fold increase in the number of BrdU$^+$ cells compared to non-treated control slices (from 8±6% to 87±5% of DAPI-positive cells after Shh-N exposure) (Fig. 6A, C, E). In contrast, no significant difference was observed in the number of BrdU$^+$ cells after exposure of mutant slices to Shh-N (from 10±4% to 13±7% after Shh-N exposure) (Fig. 6B, D, E).

To further test whether loss of Kif3a in GCPs cause cell-autonomous proliferation defects in response to Shh, GCPs were isolated from the cerebella of P3-5 *Kif3a* mutant and control mice (see Methods) and were allowed to form aggregates *in vitro* as previously described (Dahmane and Ruiz I Altaba, 1999). Addition of Shh to the serum-free culture media for

48h increased the size of the control aggregates by 2-fold on average, compared to *Kif3a* mutant (Fig. 6F-H). Thus, these observations strongly suggest that *Kif3a* is required for GCPs proliferation in response to Shh.

Loss of *Smo* in GCPs mimics the phenotype of *Kif3a* deficient mice

If the phenotype observed in the conditional *Kif3a* mutants is due to defective Shh signaling, we should expect a similar phenotype in conditional *Smo* mutants. *hGFAP: .Cre* transgenic mice were crossed to *Smo^{fl/fl}* mice (Long et al., 2001) to generate *hGFAP: .Cre; Smo^{fl/fl}* mutant mice (called conditional *Smo* mutants). Conditional *Smo* mutants showed ataxia and tremor similar to what we observed in the conditional *Kif3a* mutants. The overall size of P21 conditional *Smo* mutant brains was slightly smaller than wild-type brains (not shown). However, the cerebellum showed a pronounced reduction in size similar to that observed in the conditional *Kif3a* mutant. As the conditional *Kif3a* mice, the conditional *Smo* mutant mice had the basic layered cytoarchitecture of the cerebellum, but showed more severe defects in foliation (Fig. 7A, B). Although Shh signaling cascade was disrupted in conditional *Smo* mutant mice as no expression of Gli1 could be detected by in situ hybridization (not shown), primary cilia were still present in the GCPs (Fig. 7D). It has been shown that the extent of cerebellar foliation correlates with the level of Shh signaling (Corrales et al., 2006). Our results suggest that loss of *Smo* in GCPs results in a similar, but more severe cerebellar foliation defects compared to *Kif3a* mutation.

Kif3a is epistatic to *Smo* in cerebellar development

The more severe defects in the cerebellar development in conditional *Smo* mutants than in conditional *Kif3a* mutants suggested that some Shh signaling persisted in the absence of primary cilia. If this was the case, the removal of both *Kif3a* and *Smo* should result in a phenotype similar to that observed in conditional *Smo* mutants. However, at P25 the conditional *Kif3a* and *Smo* double mutant had a cerebellum strikingly similar to that of the conditional *Kif3a* mutant. In contrast to severe loss of foliation in conditional *Smo* mutants, the cerebellum of double mutants still retained rudimentary folia like in conditional *Kif3a* mutants (Fig. 7A-C). To further investigate the differences in the conditional *Kif3a* mutants, conditional *Smo* mutants and conditional *Kif3a* and *Smo* double mutant mice, we compared the proliferation of GCPs at E18.5. We counted the number of BrdU$^+$ GCPs in five regions along the AP axis of mutant cerebellum. The number of BrdU$^+$ cells in conditional *Smo* mutants was reduced in all five cerebellar regions studied. In contrast, in conditional *Kif3a* mutants the reduction was less severe in the posterior two lobes (compare Fig. 4E, F with Fig.7E, G). Consistent with the level of foliation, in the conditional *Kif3a* and *Smo double* mutants, the number of BrdU$^+$ cells was significantly increased compared to conditional *Smo* mutants and comparable to the level observed in conditional *Kif3a* mutants (Compare Fig. 4E, F with Fig.7). Thus, *Kif3a* is epistatic to *Smo* in cerebellar development. Altogether, our study strongly suggests that IFT protein KIF3A is required for GCPs proliferation in response to Shh and acts downstream of Smo.

Discussion

In this study, we have examined the role of primary cilia in cerebellar development and specifically on GCP proliferation. We utilized Cre recombinase under the control of the *hGFAP* promoter to generate conditional mutant mice for *Kif3a* and/or *Smo* in GCPs. We observed a severe cerebellar defect due to reduced proliferation of neuronal progenitors in these conditional mutants. Interfering with intraflagellar transport in GCPs severely disrupts their Shh-induced proliferation. The defects were similar to those observed when Smo is inactivated in this same lineage. The study therefore suggests a critical role for primary cilia in cerebellar development and a possible explanation for the hypoplasia of cerebellar vermis found in Joubert and Bardet-Biedl syndromes associated with defective primary cilia (Louie and Gleeson, 2005).

The proliferation defect of GCPs is cell-autonomous

We report here that *hGFAP*-driven expression of *Cre* is detected at E14.5 in precursor cells located in the VZ and RL of the developing cerebellum. Similar results were obtained using the endogenous mouse *GFAP* promoter, suggesting that these precursor cells express *GFAP* very early during cerebellar development (Delaney et al., 1996; Marino et al., 2000; Kwon et al., 2001). We traced the fate of these cells using conditional reporter mice and found that progenitors expressing *hGFAP*-driven *Cre* produce granule neurons. The present report shows that the granule cell lineage can be effectively targeted using *hGFAP∷Cre* mice.

Primary cilia were absent in GCPs in conditional *Kif3a* mutants but were present in the neighboring meningeal cells, supporting the specific requirement of primary cilia in GCPs. In addition, we show that inhibition of ciliogenesis in GCPs from E14.5 does not lead to any cerebellar defects until the time between E16.5 and E18.5, suggesting that the initial specification of GCPs is not dependent on the presence of the primary cilia. In contrast, significantly less proliferating cells were counted in the EGL from E18.5, suggesting that GCPs require primary cilia for the extensive expansion to make the large number of cerebellar granule cells. Conditional ablation of *Smo* in the same population of *hGFAP* expressing cells showed similar phenotypes to *Kif3a* mutants, and cilia are present in GCPs of conditional *Smo* mutants, suggesting that the absence of cilia in *Kif3a* mutants is not secondary to the cerebellar defects itself. *hGFAP* promoter target gene expression to Bergmann glia in addition to GCPs. We therefore cannot exclude that the observed disruption of cerebellar development could be due in part to loss of *Kif3a* or *Smo* in these cells. However, the proliferation defects observed in isolated GCPs from *Kif3a* mutants compared to controls provide an additional argument in favor of a cell-autonomous proliferation defect of GCPs in *Kif3a* mutants. Recent work suggests that the kinesin-II complex may also have a function in intracellular trafficking and mitosis (Brown et al., 2005; Teng et al., 2005; Haraguchi et al., 2006). However, the distributions of cell-cell adhesion molecules such as N-cadherin and β-catenin appeared normal in GCPs from *Kif3a* mutants (Supplementary Fig. 5). Moreover, we did not observe an abnormal cytokinesis nor an increase in cell death at P0, which would occur as a consequence of defective mitosis (Supplementary Fig. 4). Instead, multiple experimental evidence strongly suggest that kinesin-II has a prominent function in ciliogenesis (Kozminski et al., 1995; Morris and

Scholey, 1997; Nonaka et al., 1998; Takeda et al., 1999; Marszalek et al., 1999; Sarpal et al., 2003; Snow et al., 2004). Similarly, the GCPs in the conditional *Kif3a* mutants showed normal proliferation at E16.5 and defects begin to manifest at E18.5 coinciding with the initiation of Shh-dependent expansion of the GCPs. Our double mutant analyses also showed that *Kif3a* act downstream of *Smo*, which is not likely to occur if *Kif3a* is required for general cytokinesis or intracellular trafficking in GCPs. Furthermore, defective proliferation of GCP was also observed in other ciliary mutants, *Ift88* (Chizikoff et al., 2007) and *Ftm* mutants (this study). Thus, the simplest interpretation of the phenotypes observed in *Kif3a* mutants is that *Kif3a* is required to make primary cilia to mediate Shh signaling in GCPs. Nonetheless, we cannot completely rule out the possibility that the cerebellar phenotype observed in *Kif3a* conditional mutants could be due to other possible roles of Kif3a since it is also shown to localize to axons (Kondo et al., 1994) and synapses (Muresan et al., 1999). It is also possible that signaling other than Shh signaling is also affected in our conditional *Kif3a* mutants since 5-HT, Sst3, and PDGF receptors were also shown to localize to primary cilia (for review, see Marshall and Nonaka, 2006) although the significance of this localization *in vivo* is still lacking.

Requirement of cilia for Shh signal transduction

It was recently demonstrated that primary cilia play an essential role in Shh signaling. Defective primary cilia cause diverse defects including neural tube patterning defects and polydactyly, which resemble those occur as a consequence of Shh signaling defects (Huangfu et al., 2003; Huangfu and Anderson, 2005; Liu et al., 2005; Haycraft et al., 2005; May et al., 2005). Consistently, Smo localizes to primary cilia in response to Shh, and IFT function and primary cilia are required for Smo activity (Corbit et al., 2005; May et al., 2005).

In this study, we provide the first evidence that the primary cilium is essential for Shh-induced proliferation of GCPs. Defects in proliferation were first detected in conditional *Kif3a* mutants around E18.5, which coincides with the first expression of *Shh* in Purkinje cells (Corrales et al., 2004; Lewis et al., 2004). Inhibition of ciliogenesis in GCP leads to a dramatic decrease in two positive readouts of Shh activity, the expression of *Gli1* and *cyclin D1* mRNA, in these cells. Consistently, although Shh elicited a proliferative response in isolated GCPs and GCPs in slices made from wild-type, it was unable to induce GCP proliferation from conditional *Kif3a* mutants. Interestingly, *Shh* expression was increased in the large cell fraction enriched in Purkinje cells in conditional *Kif3a* mutants compared with controls. Although the large cell fraction might contain other cell types like Bergmann glia or astrocytes, these cells do not express *Shh* (Dahmane and Ruiz I Altaba, 1999; Lewis et al., 2004). These results thus suggest that *Shh* expression by Purkinje cells might be in part regulated by GCPs. The transcription factors acting upstream in the pathway that control *Shh* expression in the cerebellum are unknown, but six *Shh* enhancers that control *Shh* expression in the ventral neural tube have recently been identified (Jeong et al., 2006). The level of Shh signaling is directly linked to the complexity of the cerebellar foliation pattern (Corrales et al., 2006). Furthermore, inappropriate Shh pathway activation in the cerebellum can cause medulloblastomas (Goodrich et al., 1997; Oliver et al., 2005) and the inhibition of the Shh signaling pathway can arrest the growth of a number of brain tumors (Romer et al.,

2004; Sanchez and Ruiz i Altaba, 2005). For all these reasons it is crucial to better understand how *Shh* expression in Purkinje cells is regulated. Our observations suggest that Purkinje cells increase Shh production when the number of GCPs is reduced.

In the absence of *Smo*, the rate of proliferation of GCPs at E18.5 is reduced significantly more than that in conditional *Kif3a* mutants. The cerebellar foliation in conditional *Smo* mutants were also more severely disrupted than that in conditional *Kif3a* mutants. Previous studies in spinal cord and limb developments showed that both activator and repressor functions of Gli proteins require primary cilia (Haycraft et al., 2005; Huangfu et al., 2005; Liu et al., 2005; May et al., 2005). Thus, less Gli repressor activity in conditional *Kif3a* mutants than in conditional *Smo* mutants could result in less severe phenotype in conditional *Kif3a* mutants. It was recently shown that removing one copy of Gli3 in *Shh* null mutants partially rescued the structure of the cerebellum at E18.5 (Blaess et al., 2006). Alternatively, the less severe phenotype in conditional *Kif3a* mutants could be due to some Shh signaling occurring independently of primary cilia. In *Drosophila*, where sensory neuron and sperm are the only ciliated cells, mutations disrupting ciliogenesis do not affect Shh signaling (Han et al., 2003; Sarpal et al., 2003). We tested these two possibilities by examining the phenotype of the conditional *Kif3a* and *Smo* double mutant. This double mutant showed a very similar phenotype to that of the conditional *Kif3a* mutant, thus supporting the first possibility. We, therefore, suggest that in conditional *Smo* mutants, a cilia dependent-repressor activity (probably Gli3) remains, leading to a more severe proliferation defect, whereas in conditional *Kif3a* mutants or conditional *Kif3a* and *Smo* double mutants, the loss of repressor (Gli3) as well as activators (Gli1, 2) leads to a less severe proliferation defects. It would be interesting to directly address this question by comparing Gli3 processing in GCPs from *Kif3a, Smo* and *Kif3a* and *Smo* double mutants.

Connection between cilia and Joubert syndrome

Conditional *Kif3a* mutant mice display cerebellar vermis hypoplasia very similar to that observed in Joubert syndrome and in Bardet-Biedl syndrome (Louie and Gleeson, 2005). In these patients, a diminished density of granule neurons has been reported, which could be due to proliferation defects of GCPs (Joubert et al., 1969). Genetic analysis of these patients has identified mutations in 3 families of genes (nephrocystin, Bardet-Biedl syndrome and AHI1), several of which encodes proteins that have been localized to cilia and/or implicated in ciliary function and assembly. In addition, some of these genes are expressed in the cerebellum and are required for cerebellar development. First, the *AHI1* gene encoding the Jouberin protein, whose function is still unknown, is expressed at a maximum level in the mouse cerebellum at birth (Dixon-Salazar et al., 2004). Structural studies of the Jouberin protein suggest that it may interact with nephrocystin-1 protein, which is localized to primary cilia (Otto et al., 2003). Second, the *nephrocystin-6* gene is first expressed around E18 and is superimposable to the *Gli1* expression pattern in the developing murine cerebellum (Valente et al., 2006). Moreover, nephrocystin-6 protein is localized to the centrosome and to the base of cilia, and abrogation of its function in zebrafish recapitulates the cerebellar phenotype of Joubert syndrome (Sayer et al., 2006; Valente et al., 2006). Finally, the *nephrocystin-8* gene (also called *Rpgrip1l* or *Fantom*) has recently been identified to be mutated in Joubert syndrome (Delous et al., 2007; Arts et al., 2007). Most

importantly, mutant mice in which *nephrocystin-8* gene has been inactivated showed cerebellar hypoplasia and defects in GCPs proliferation similar to *Kif3a* mutants (Delous et al., 2007; this study).

These results are consistent with our findings that cilia play a crucial role in cerebellar development and specifically in Shh-induced proliferation of GCPs. To further understand the pathogenesis in Joubert syndrome, it would be interesting to test whether *AHI1, nephrocystin-6* and *nephrocystin-8* gene expression are controlled by Shh signaling and/or how these proteins interact with downstream effectors of Shh signaling pathway in primary cilia. It remains intriguing why key signaling processes, as those mediated by Shh, require this minute hair-like extension from the surface of the cells. Primary cilia may act as an antenna (Singla and Reiter, 2006) to concentrate relevant receptors on a small surface of the cell, thereby allowing signaling to reach the threshold required for activation of signal transduction. Placing receptors and signaling components in a thin protrusion at one point in the cell may help the cells extend its reach into the extracellular space, and orient the cellular response with respect to an external stimulus. Recent work highlights the importance of primary cilia in multiple processes including cell specification in CNS. The present work extends this view by showing how this primary cilium is required for the proliferation of an important neuronal progenitor and the development of an entire brain structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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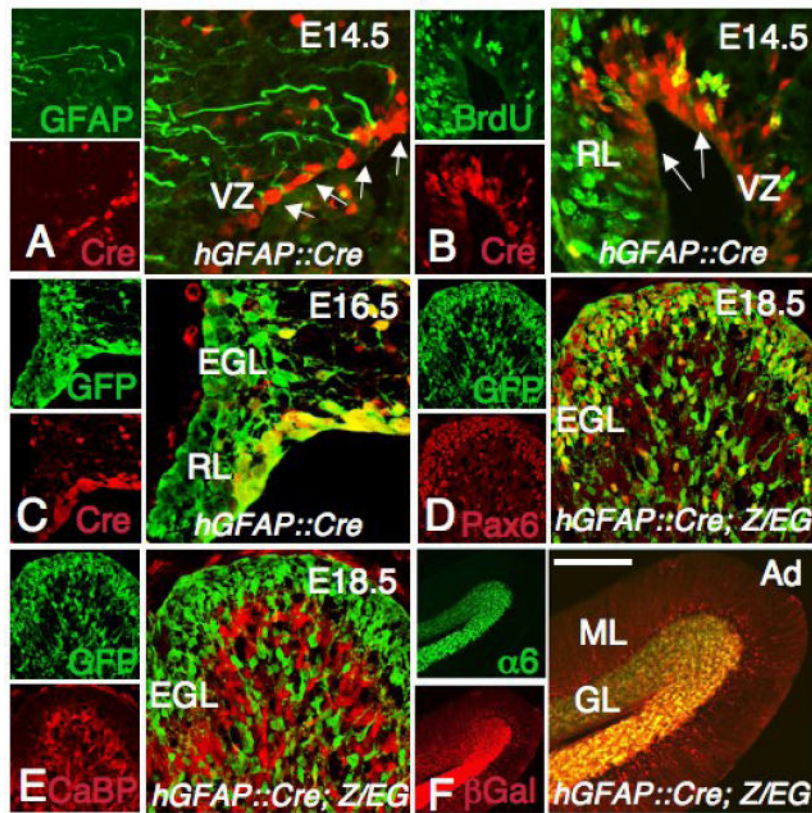
Fig. 1. The granule cell lineage is recombined by the *hGFAP::Cre* transgene
(A-F) Sagittal sections of the cerebellum from a *hGFAP::Cre* (A-B), *hGFAP::Cre; Z/EG* (C-E) and *hGFAP::Cre; R26R* (F) mice at E14.5 (A-B), E16.5 (C), E18.5 (D-E) and adult (Ad, F) stained with the antibodies shown in each panel. (A, B) Cre immunoreactivity is detected in proliferating GFAP$^+$ cells in the ventricular zone (VZ) and the RL (arrows in A, B). (C) At E16.5, Cre$^+$ cells are still present in the ventricular zone and in the RL but are not observed in the EGL, which already contains progeny of Cre$^+$ cells (GFP$^+$). (D, E) At E18.5, Cells derived from Cre$^+$ cells (GFP$^+$) are localized in the EGL and inner layers, and express the GCP marker Pax6 (D), but not the Purkinje cell marker CaBP (E). (F) In the adult, Bergmann glial fibers in the ML and α6$^+$ granule cells are β-galactosidase$^+$. Scale bars: 50 μm (A, B), 30 μm (C), 100 μm (D, E) and 300 μm (F).
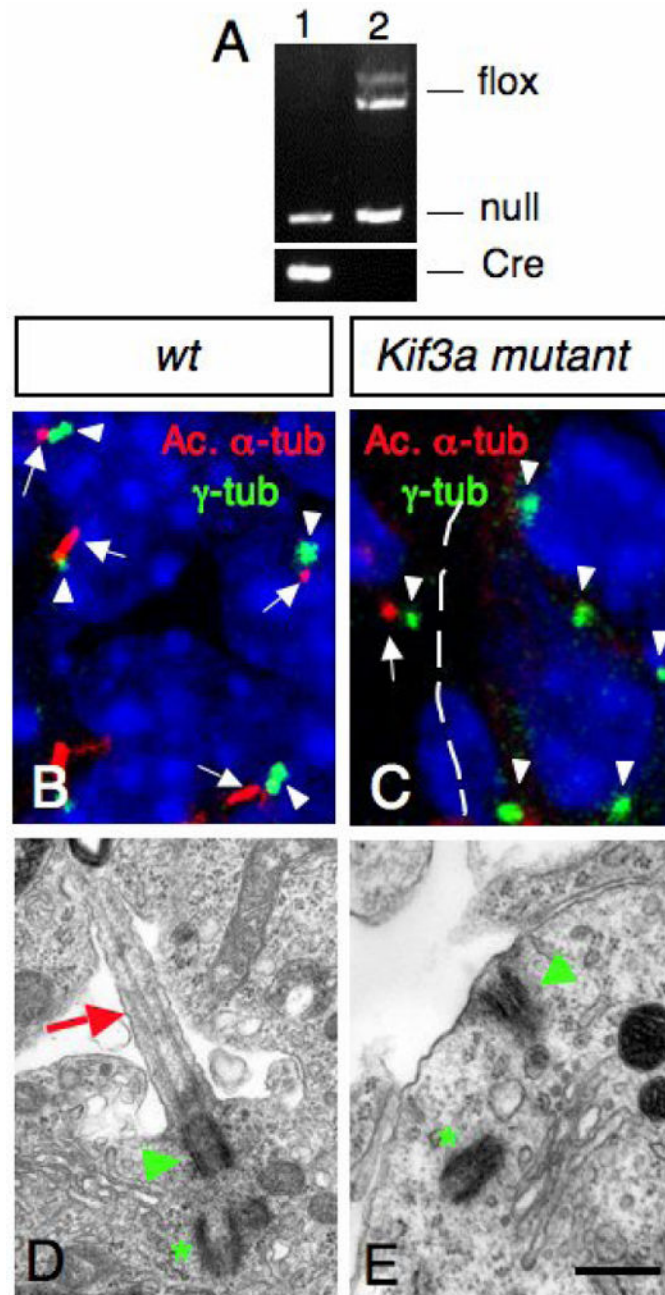
**Fig.2. Lack of *Kif3a* inhibits ciliogenesis in the EGL**

(A) PCR products obtained after amplification of genomic DNA of purified GCPs from *hGFAP::Cre; Kif3a^{fl/-}* (lane 1) and *Kif3a^{fl/-}* (lane 2) cerebellum. Note that the LoxP band is absent in purified GCPs from *hGFAP::Cre^+* mice (lane 1). (B, C) Confocal projections of double immunostaining with an anti-acetylated α-tubulin antibody (red, arrow) and an anti-γ-tubulin antibody (green, arrowhead) on sections of wild type (B) and conditional *Kif3a* mutant (C) at E18.5. In control animals, most GCPs extend a primary cilium (arrows) from a basal body (arrowheads). These cilia were absent in the EGL in the conditional *Kif3a* mutant. Basal bodies are still present in the mutant EGL (arrowhead). Note that the mutation

does not affect meningeal cells since ciliary staining could still be observed in this structure (arrow in C). (D, E) Electron micrographs of GCPs from wild type and conditional *Kif3a* mutants at P0. (D) In wild type animals, a primary cilium (red arrow) extends from a basal body (green arrowhead) nearby a centriole (green asterisk). (E) In conditional *Kif3a* mutant a basal body (green arrowhead) is attached to the cell membrane nearby a centriole (green asterisk), but the primary cilium is absent. 5 μm (B-C), 0.5 μm (D-E).
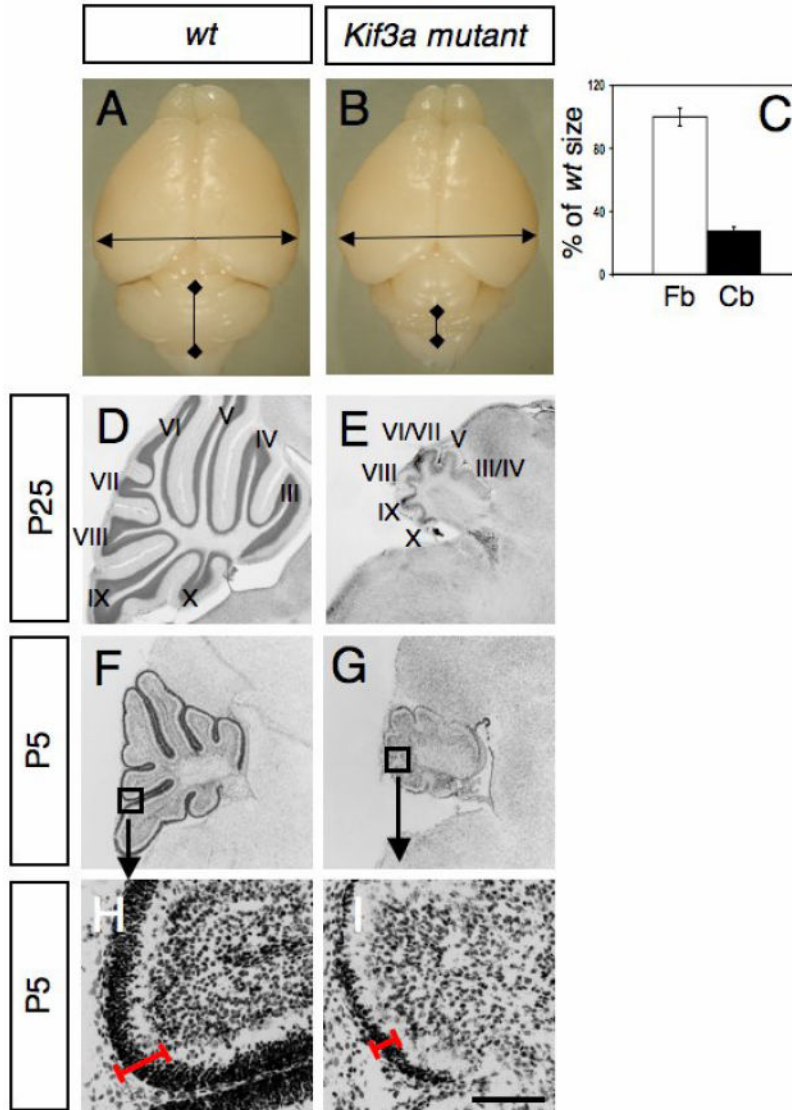
Fig. 3. Lack of *Kif3a* severely disrupts cerebellar development
(A-B) Gross appearance of a brain from a wild-type littermate (A) and a conditional *Kif3a* mutant (B) at P25. (C) The size of the forebrain (Fb) and cerebellum (Cb) from wild-type and conditional *Kif3a* mutant were measured as indicated by arrows in A, B. Although the forebrain from conditional *Kif3a* mutants is not significantly different from wild-type, the size of the cerebellum is dramatically reduced. (D-I) Cresyl violet staining of sagittal sections of cerebellum from wild type (D, F, H) and conditional *Kif3a* mutant (E, G, I) cerebellum at P25 (D-E) and P5 (F-I). (F-I) Lack of *Kif3a* limits the expansion of the EGL. (H-I) High magnifications of the squares shown in (F,G) illustrate that EGL is thinner in the mutants (red line). Scale bars: 3mm (A, B), 800 μm (D-G), 130 μm (H, I).
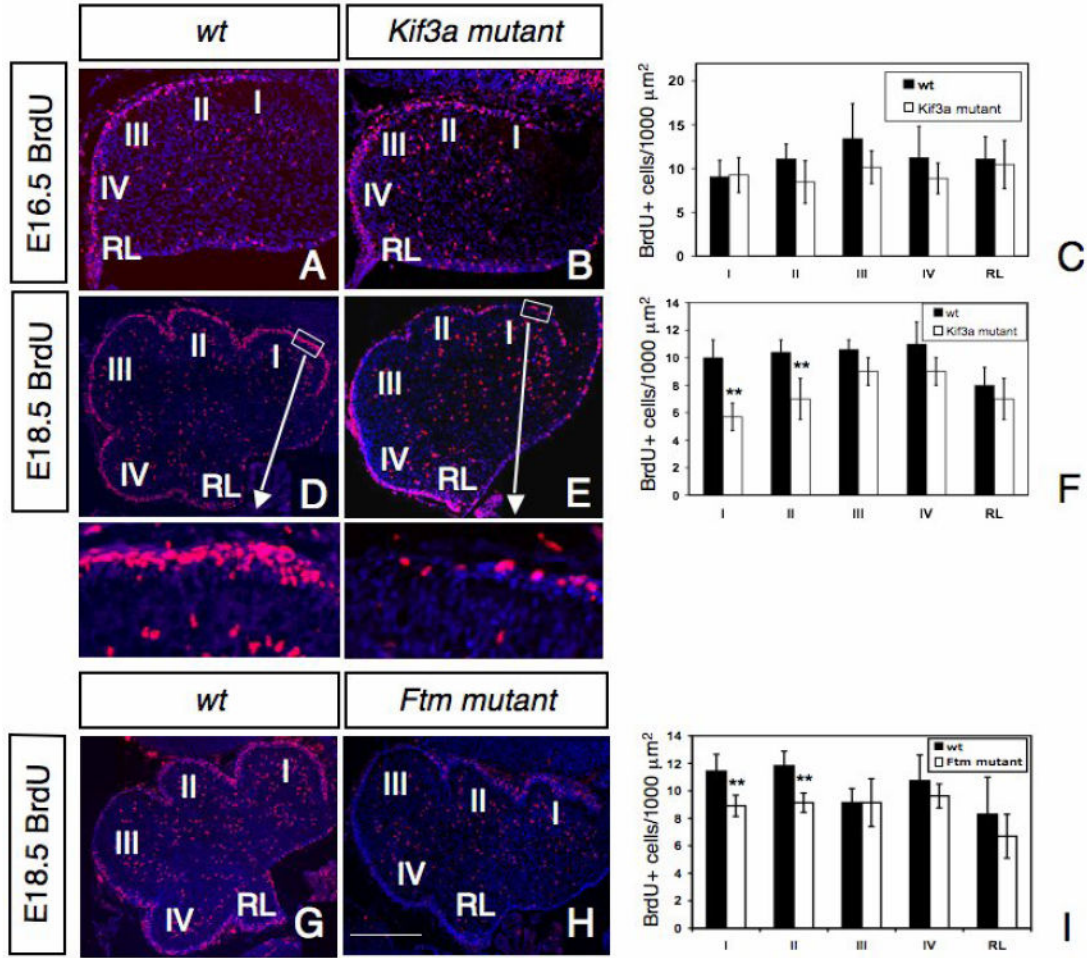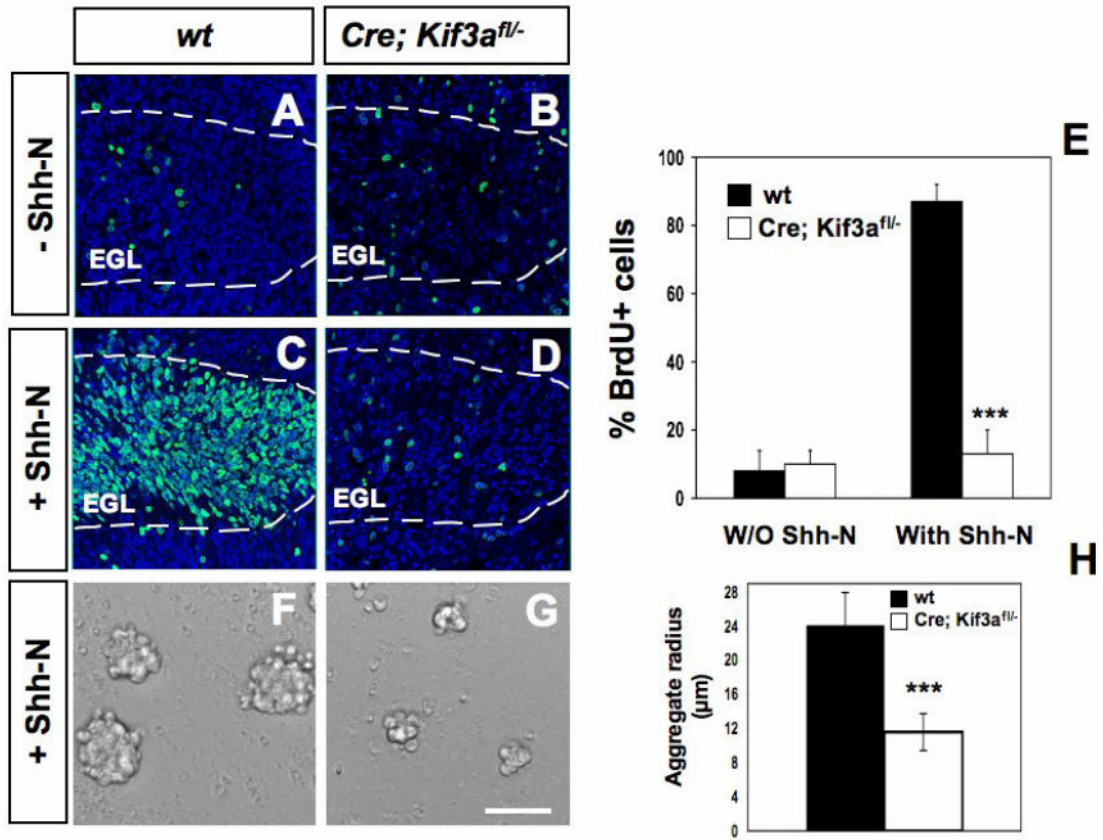
Fig. 4. Abnormal proliferation of GCPs in conditional *Kif3a* and *Ftm* mutant mice
BrdU staining of a control (A, D, G), a conditional *Kif3a* mutant (B, E) and a *Ftm* mutant (H) cerebellum at E16.5 (A-B) and E18.5 (D-E, G-H). Rostral is to the right. Squares in D, E are shown at higher magnifications below. (C, F, I) Quantification of BrdU$^+$ cells per 1000 μm^2 in regions I to IV and in the RL. Note that at E18.5, the number of BrdU$^+$ cells is much smaller in the rostral part of the cerebellum in conditional *Kif3a* and *Ftm* mutants compared to wild type, while no significant difference was observed in caudal regions of conditional *Kif3a* and *Ftm* mutants. The BrdU$^+$ cells were counted from at least three sections from each mouse at comparable mediolateral levels. Data from three mice per group were pooled for statistical analysis with Student's *t*-test. **: p<0.01. Scale bars: 100 μm (A, B), 200 μm (D-E, G-H).
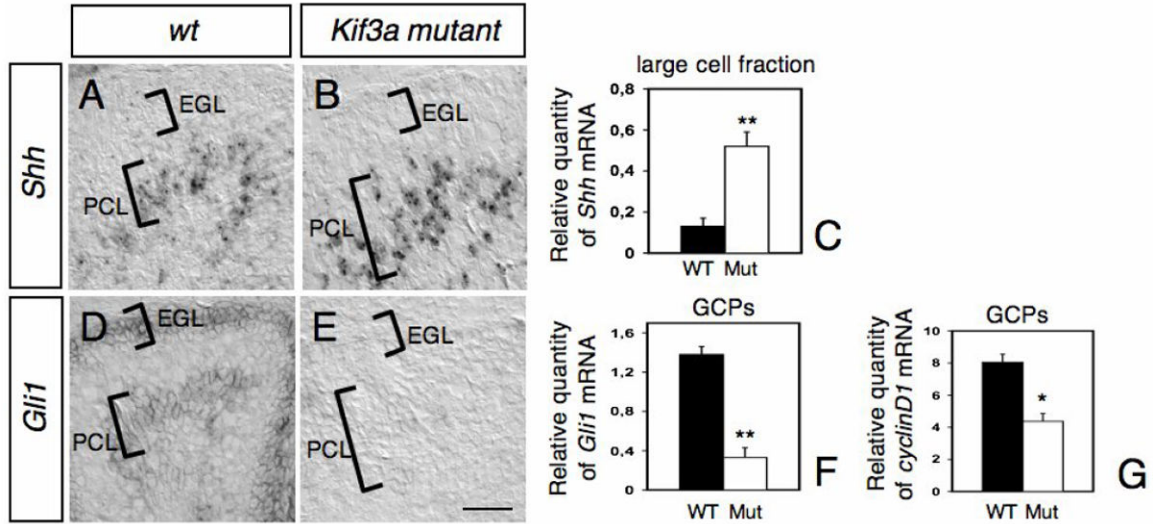
Fig. 5. Abnormal expression of *Shh* and Shh target genes, *Gli1* and *cyclin D1*, in conditional *Kif3a* mutants

In situ hybridization on sagittal sections of wild type (A, D) and mutant (B, E) cerebellum at P2 with antisense riboprobes specific for *Shh* (A-B) and *Gli1* (D-E). The location of the EGL and PCL are indicated on each panel. Note that whereas *Shh* expression is increased in the PCL of mutant compared to wild-type (A-C), *Gli1* was no longer detected neither in the EGL nor the PCL in the mutant (E). (C, F-G) Levels of *Shh* mRNAs in the large cell fraction enriched in Purkinje cells (C), and levels of *Gli1* (F) and *cyclin D1* mRNAs in GCPs (G) at P2-4 were evaluated by semi-quantitative real-time RT-PCR and are shown as the relative quantities normalized to the level of *hprt* mRNA expression. The level of *Shh* mRNA in conditional *Kif3a* mutant is significantly higher than that in the wild-type, whereas the levels of *Gli1* and *cyclin D1* mRNAs in the mutant are significantly lower than those in the wild-type. Each column and the vertical line represent the mean± SEM. Data from three mice per group were pooled for statistical analysis with Student's *t*-test. *: $p<0.05$; **: $p<0.01$. Scale bar: 80 μm.

Fig. 6. Abnormal Shh-induced proliferation of GCPs in cerebellar cultures from conditional *Kif3a* mutants

BrdU staining on cerebella sagittal slices from P3 wild type (A, C) and mutant mice (B, D) cultured with no stimulus (A–B) or 3 μg/ml Shh-N (C–D). BrdU staining (green) was counterstained with DAPI (blue) and visualized using confocal microscopy. (E) Quantification of the percentage of BrdU$^+$ and DAPI$^+$ nuclei in wild type and conditional *Kif3a* mutant slices with and without Shh-N. (F–G) Proliferation of isolated GCPs in aggregates obtained from P1 *Kif3a* mutants (G) compared to controls (F) with Shh-N for 48h. (H) Quantification of aggregate radius in wild type and conditional Kif3a mutants after 48h. Data from three mice per group were pooled for statistical analysis with Student's *t*-test. ***: p<0.001. Scale bar: 100 μm.
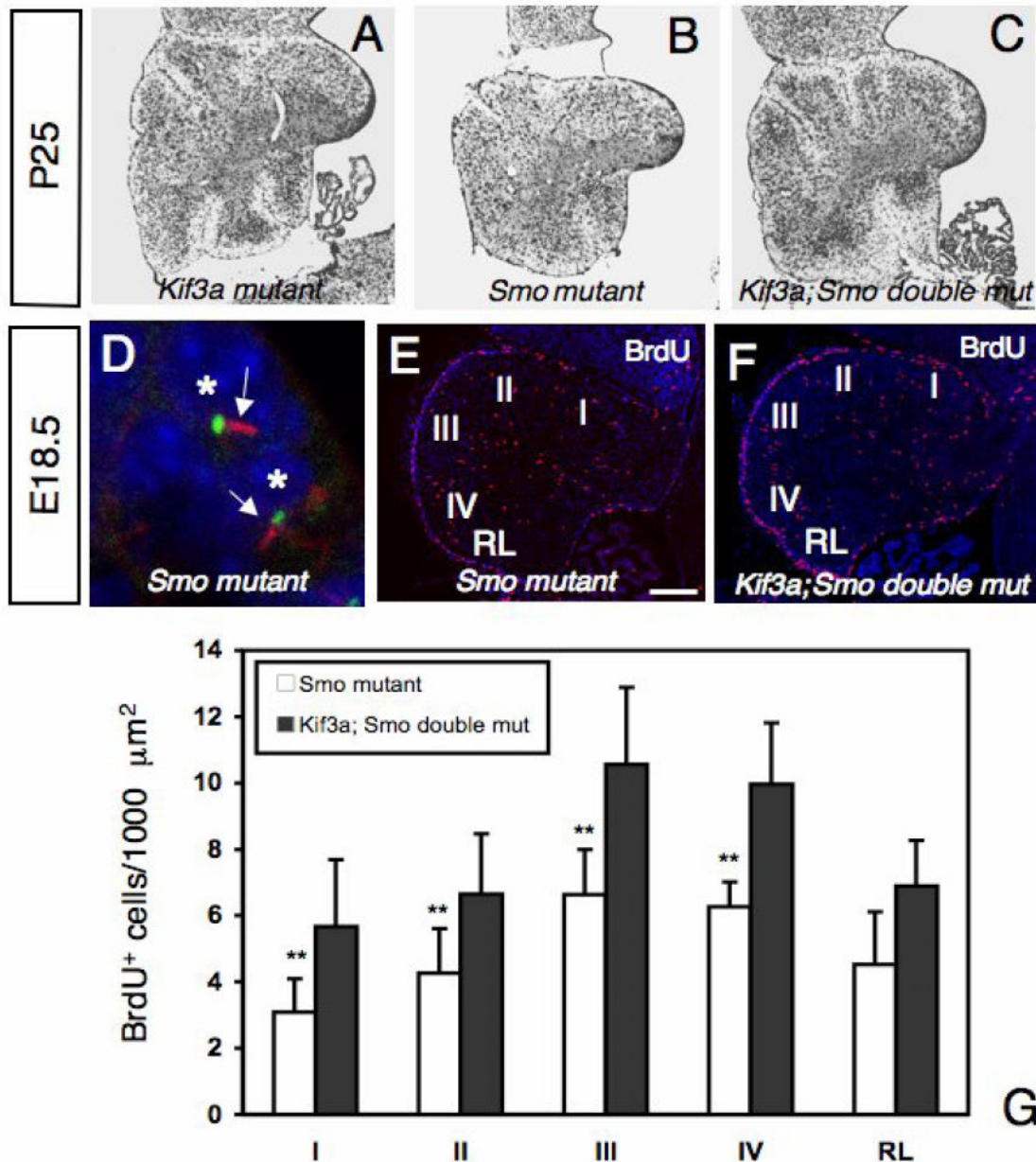
Fig.7. Abnormal proliferation of GCPs in conditional *Smo* mutant and conditional *Kif3a* and *Smo* double mutant mice

(A-C) Cresyl violet staining of sagittal sections of cerebellum from conditional *Kif3a* mutant (A), conditional *Smo* mutant (B), and conditional *Kif3a* and *Smo* double mutant cerebellum at P25. Rostral is to the right. (D) Double immunostaining with an anti-acetylated α-tubulin antibody (red) and an anti-γ-tubulin antibody (green) on sections of conditional *Smo* mutant at E18.5. In conditional *Smo* mutants, most GCPs extend a primary cilium (arrow) from a basal body. Each nucleus associated with a primary cilium is indicated by an asterisk. (E-F) BrdU staining of a conditional *Smo* mutant (E) and a conditional *Kif3a* and *Smo* double mutant (F) cerebellum at E18.5. Rostral is to the right. (G) Quantification of BrdU+ cells per 1000 μm^2 in regions I to IV and in the RL. Note that the number of BrdU+ cells is much

smaller in conditional *Smo* mutants than in conditional *Kif3a* and *Smo* double mutants, while no significant difference was observed between conditional *Kif3a* mutants and conditional *Kif3a* and *Smo* double mutants (compare with Fig.4F). The BrdU^{+} cells were counted from at least three sections from each mouse at comparable mediolateral levels. Data from three mice per group were pooled for statistical analysis with Student's *t*-test. **: p<0.01. Scale bar: 230 μm (A-C), 2 μm (D) and 100 μm (E-F).