The G-protein Alpha Subunit Gsα Is A Tumor Suppressor In Sonic Hedgehog-driven Medulloblastoma

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Abstract

Medulloblastoma, the most common malignant childhood brain tumor, exhibits distinct molecular subtypes and cellular origins. Genetic alterations driving medulloblastoma initiation and progression remain poorly understood. Herein, we identify GNAS, encoding the G-protein Gsα, as a potent tumor suppressor gene that defines a subset of aggressive Sonic Hedgehog (Shh)-driven human medulloblastomas. Ablation of the single Gnas gene in anatomically-distinct progenitors is sufficient to induce Shh-associated medulloblastomas, which recapitulate their human counterparts. Gsα is highly enriched at the primary cilium of granule neuron precursors and suppresses Shh-signaling by regulating both the cAMP-dependent pathway and ciliary trafficking of Hedgehog pathway components. Elevation of a Gsα effector, cAMP, effectively inhibits tumor cell proliferation and progression in Gnas mutants. Thus, our gain- and loss-of-function studies identify a previously unrecognized tumor suppressor function for Gsα that acts as a molecular link across Shh-group medulloblastomas of disparate cellular and anatomical origins, illuminating G-protein modulation as a potential therapeutic avenue.

Keywords

medulloblastoma; G-protein; cAMP; GPCR; cell lineage; sonic hedgehog signaling; cilia; cellular origins

Medulloblastoma (MB) comprises clinically and molecularly distinct that arise either in the cerebellum or brainstem. Although current treatments improve survival rates, patients suffer severe side effects and relapse of tumors carrying resistance mutations, underscoring an urgent need for alternative targeted therapies. Deregulation of G-protein coupled receptor (GPCR) pathways has been implicated in medulloblastoma, however, underlying signal transduction events that drive tumor initiation and progression remain obscure. GNAS encodes the heterotrimeric Gs protein alpha-subunit (Gsα) that functions as a molecular switch to transmit various GPCR signals to control cell growth, survival, and motility. Recent genome-wide analyses of somatic mutations in cancers identified GNAS as one of the most frequently mutated genes. Although most somatic tumor types acquire gain-of-function GNAS mutations, analysis of a copy number database (Tumorscape, Broad Institute) surprisingly reveals that MB displays a significant loss of the chromosomal region containing GNAS (Supplementary Fig. 1) compared to other cancers. Furthermore, a recent case report showed that a 14-month-old infant with a novel homozygous nonsense mutation
within the GNAS coding region developed MB. Herein, we tested whether deregulation of Gsα-coding GNAS may contribute to MB formation.

Results

**GNAS defines a subset of aggressive SHH-group tumors**

Human MB can be classified into at least four principal subgroups, namely, WNT (Wingless) group, SHH (Sonic hedgehog) group, group 3 and group 4, based on distinct gene expression profiles. To define the correlation of GNAS in MB subgroups, we examined GNAS expression from two independent, non-overlapping patient cohorts in the Boston and Heidelberg series. We found that low GNAS expression was tightly correlated with significantly decreased overall survival within SHH-group tumors (SHH-MB), which comprise approximately 30% of all MBs (Fig. 1a,b). Notably, the prognostic impact of GNAS was not observed in other group tumors and across MB subgroups (Fig. 1c,d; Supplementary Figs. 2,3). These observations suggest that low expression or loss of GNAS specifically defines a subset of aggressive SHH-group MBs.

**Loss of Gnas in neural stem/progenitor cells induces MB formation with full penetrance**

To determine whether Gnas inactivation could lead to brain tumorigenesis, we deleted Gnas in neural stem/progenitor cells by breeding floxed Gnas mice (Gnaslox/lox) with an hGFAP-Cre line. Strikingly, all resulting hGFAP-Cre+/−:Gnaslox/lox conditional knockout mice (designated as GFAP:Gnas) developed MB-like tumors at adult stages (Fig. 2a,b). We observed expansion of granule neuron progenitors (GNPs) in the cerebellar external granular layer (EGL) beginning at neonatal stages, when the control cerebellum contained only a few rows of GNPs on its surface. Diffuse, continuous GNP expansion continued to increase at postnatal stages. Gnas mutant cerebella were exophytic and delineated by a thick and disorganized EGL (Fig. 2a). By six weeks, GFAP:Gnas mice developed a diffuse MB-like tumor exhibiting the densely-packed, “small round blue” GNP-like histology (Fig. 2c; inset), resembling the histological features of human MB. In the mutants at P60, the neoplastic cells were highly proliferative as indicated by extensive expression of Ki67, a proliferative marker, which was barely detectable in controls (Fig. 2d). 100% of animals succumbed to the tumor around 3-4 months of age (Fig. 2e). Although the hGFAP-Cre-recombined cells appear in most brain regions, tumor formation was confined only to the cerebellum during the lifespan of Gnas mutants.

To ascertain gene expression alterations caused by Gnas loss, we examined mRNAs isolated from the cerebella of control and GFAP:Gnas mice at P60 by RNA-deep sequencing. In tumors of Gnas mutants, our data revealed an up-regulation of Shh signaling pathway components (Fig. 2f). Quantitative RT-PCR confirmed that expression of Shh target genes and pathway components was significantly up-regulated (Fig. 2g). Consistently, mRNA in situ hybridization revealed intense expression of Shh downstream genes including Gli1, Gli2, Ptc1 and cyclinD1 (Cnd1) (Fig. 2h). Furthermore, we observed significant elevation of Shh direct target genes Gli1 and Ptc1 in GNP-like tumor cells compared with normal GNPs, suggesting a cell-intrinsic effect of Gnas mutation on Shh signaling activation. By contrast, expression of Wnt-target genes was not substantially altered.

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Supplementary Fig. 4), consistent with previous findings that Gnas loss does not affect Wnt signaling in other cellular systems. We observed widespread expression of GNP markers Zic1 and Atoh1 (a.k.a. Math1), along with Shh-regulated targets including Olig2, but very few astrocytic GFAP+ astrocytes (Fig. 2j). Thus, Gnas loss results in an increase or alternatively a de-repression of physiological levels of Shh pathway activity and over-proliferation of GNP-like tumor cells.

**Gsα activity suppresses hedgehog signaling**

To test whether the GTPase activity of Gsα protein is required for inhibition of Shh signaling, we treated GNP cells isolated from wildtype neonates with NF449, a selective Gsα antagonist, which prevents GTP binding to Gsα and blocks Gsα GTPase activity. Treatment of NF449 resulted in a significant up-regulation of Shh target genes Gli1, Gli2, Ptc1 and Myc-N and caused a decrease of cAMP levels (Fig. 3a), suggesting that inhibition of Gsα GTPase activity activates Shh signaling. To investigate the effect of Gsα gain-of-function activity on Shh signaling, we generated a constitutively activated form of Gsα, GsαQ227L (GsCA), which resulted in a GTPase-defective, active GTP-bound Gsα protein. Overexpression of GsCA in GNPs suppressed the upregulation of Shh targets Gli1, Ptc1, Myc-N and Ccnd1 induced by a Shh agonist SAG (Fig. 3b), indicating that Gsα activation inhibits hedgehog signaling.

**Elevation of the Gsα effector cAMP inhibits MB growth**

The classic signal transduction pathway of Gsα is through activation of adenylyl cyclase, which, in turn, produces intracellular cAMP. cAMP has been shown to activate cAMP-dependent Protein Kinase A (PKA), a negative effector of Shh signaling. Tumor cells isolated from Gnas mutants had a significant reduction in intracellular cAMP levels, while treatment with the adenylyl cyclase agonist forskolin (FSK) elevated cAMP levels (Fig. 3c). To test the hypothesis that cAMP elevation could inhibit Shh signaling activation, we treated Gnas mutant GNPs with Rolipram, which elevates cAMP levels by selectively inhibiting phosphodiesterase-4 activity to block cAMP degradation, FSK or a non-hydrolysable cAMP analog db-cAMP. Each of these cAMP-raising agents significantly reduced expression of Gli1 and Ptc1 (Fig. 3d). In addition, in wildtype GNPs, treatment with FSK and Rolipram enhanced the proteolytic processing of full-length Gli3 into a repressive form Gli3R (Fig. 3d), an inhibitor of Shh target expression. In contrast, inhibition of cAMP-dependent PKA with two different small molecule inhibitors, H89 and KT570, significantly increased expression of Gli1, Ptc1 and Ccnd1 (Fig. 3e). Furthermore, mitigation of PKA activity by H89 (Supplementary Fig. 5) can restore Shh target expression suppressed by constitutively activated Gsα (Fig. 3f). Thus, our data are in keeping with previous observations that Gsα-mediated signaling can elevate cAMP levels to inhibit hedgehog target gene expression through cAMP-dependent PKA activity in other cellular systems.

To determine the effects of cAMP elevation on tumor growth in vivo, we evaluated the efficacy of Rolipram, which is well tolerated and readily crosses the blood brain barrier in vivo. Control and GFAP:Gnas mutants at the young adult stage P35 were randomized to receive either daily i.p. vehicle or Rolipram for 30 days with a dosage exhibiting effective...
anti-tumor activity in vivo, and assessed for tumor development. Rolipram administration during this period did not affect overall cerebellar structure and myelin formation (Fig. 3g; Supplementary Fig. 6). Vehicle-treated Gnas mutants displayed extensive tumor cell expansion and bulged cerebella (Fig. 3g). In contrast, in Rolipram-treated mutants, the tumor size and proliferation of Zic1+ GNP-like tumor cells were substantially reduced (Fig. 3g-i). In addition, Rolipram treatment exhibited a significantly extended lifespan of Gnas mutants (Fig. 3j). Thus, elevation of the Gsα effector cAMP by Rolipram could lead to inhibition of tumor growth in Gnas mutants.

Gsα controls ciliary trafficking of hedgehog signaling components in GNPs

Ciliary trafficking of signaling components has an important role in regulating Shh pathway activity and MB formation. Strikingly, we observed that Gsα was highly enriched at the primary cilium of GNPs (Fig. 4a) but hardly detectable in mouse embryonic fibroblasts (Supplementary Fig. S7), suggesting a unique ciliary function of Gsα for GNP development. In the presence of Shh, ciliary localization of Gsα is diminished (Fig. 4a,b). Treatment of Shh and its agonist SAG did not alter the total amount of Gsα protein, but rather reduced the amount of the GTP-bound, active form of Gsα protein (Gsα-GTP; Supplementary Fig. 8).

In GNPs from GFAP:Gnas mutants, Gsα expression was essentially depleted, while total levels of Smoothened (Smo) were comparable to controls (Supplementary Fig. 9). At the tips of cilia, approximately 64.3 ± 6.2% cells exhibited strong immunoreactivity for Gli2, a Shh downstream effector (Fig. 4c, insets), whereas Gli2 was hardly detectable or weakly presented in control GNPs (Fig. 4c,d). Consistent with a principal function of PKA in restraining Gli2 activation, our observation suggests that Gnas loss reduces cAMP-dependent PKA activity and increases Gli2 accumulation at ciliary tips, leading to Shh signaling activation. We further detected ciliary translocation of Smo seven-transmembrane protein in the majority of Gnas mutants, but not in control GNPs, where Smo was diffusely localized in the cytoplasm near the base of cilia but absent from ciliary shafts (Fig. 4e,g). Conversely, a Smo-inhibiting protein, Ptc1, was mainly detected in the ciliary shaft of control GNPs (Fig. 4f) but absent from cilia in Gnas mutants (Fig. 4f,g). In contrast, ciliary trafficking of a Shh signaling regulator Gpr161 was not altered in Gnas mutants (Supplementary Fig. 10), suggesting a specificity of Gsα in regulating ciliary localization of GPCRs. Consistent with the existence of multiple Gsα-mediated GPCRs, Gpr161−/− embryos exhibited milder developmental defects than Gnas−/− animals. Given that PKA null mutation could lead to Gli2 accumulation at the tips of cilia, but not the ciliary trafficking of Smo in neural progenitors, our observations of both strong Gli2 signal at the ciliary tip and Smo ciliary translocation in Gnas mutants suggest that Gsα might have an additional role in regulating Smo trafficking in primary cilia independent of cAMP-dependent PKA activity.

cAMP elevation and Smo inhibition augment suppression of tumor cell proliferation

Our data indicate that Gsα suppresses Shh signaling not only by stimulating intracellular cAMP levels to activate PKA, but also perhaps independently by inhibiting Smo activation. Treatment of Gnas mutant GNPs with GDC-0449, which blocks SMO activation induced by
SAG (Supplementary Fig. 11),\(^3\) led to a reduction in Gli1 and Ptc1 expression (Fig. 4h). Combined treatment of GDC-0449 and Rolipram however resulted in further inhibition of Shh target expression (Fig. 4h). Since Gnas mutant GNPs are highly proliferative (Fig. 4i), we then determined whether cell proliferation is responsive to cAMP elevation and Smo inhibition. The proliferation rate was reduced in Gnas-mutant GNPs treated with Rolipram or GDC-0449 to a certain extent, where Rolipram exhibited a relatively stronger effect than GDC-0449 (Fig. 4i,j). However, combinatorial treatment of both drugs was found to cause a greater inhibition of cell proliferation in Gnas mutants (Fig. 4i,j).

**Loss of Gnas in Atoh1\(^+\) or Olig1\(^+\) progenitors leads to anatomically distinct MBs**

SHH-driven MBs may arise from multiple cellular origins in human patients.\(^3\) hGFAP-Cre mediated Gnas deletion might affect multiple progenitor populations in the posterior fossa. To examine whether Gnas loss in committed GNPs could result in MB formation, we crossed Gnasfloxed mice with an Atoh1-Cre line, which directs Cre expression in GNPs of the cerebellum and dorsal brainstem cochlear nuclei.\(^3,5\) The resulting Atoh1-Cre\(^+/\!\!-\):Gnas\(^lox/lox\) (Atoh1:Gnas) mutant mice developed MB-like tumors with an expansion of tumor cells in the EGL layer (Fig. 5a). Tumor cells expressed neuronal markers Tuj1 and Zic1 extensively with few Olig2 and GFAP-expressing glial cells (Fig. 5b), and exhibited a significant up-regulation of Shh signaling target genes Ptc1, Gli1 and Hhip (Fig. 5c). This suggests that ablation of Gnas selectively in committed cerebellar GNPs is sufficient to cause Shh-associated MB formation.

To test whether other Shh-responsive progenitor cells were susceptible to oncogenic transformation due to Gnas loss, we ablated Gnas in the progenitors expressing a Shh-regulated gene Olig1.\(^3,6,7\) During embryogenesis, Olig1 is mainly expressed in the specified progenitors of the brainstem around rhombomeres r2-r4, which can give rise to oligodendrocyte precursors (OPC) and granule neuron lineage cells.\(^3,7,8\) (Fig. 5d). The Olig1\(^+\) progenitors were mainly detected in the rostral brainstem but separated from caudal brainstem progenitors in rhombomeres r6-r8, a source of Wnt-associated MBs.\(^3,9\) (Fig. 5d). They were essentially undetectable in the upper rhombic lip, EGL and cochlear nucleus (Fig. 5d; Supplementary Fig. 12), the other sources for Shh-associated MBs.\(^3,5\) Lineage tracing analysis indicated that the progeny of Olig1\(^+\) progenitors were present in a population of Zic1\(^+\) GNPs in the dorsal brainstem (Fig. 5e). Notably, Olig1-Cre\(^+/\!\!-\):Gnas\(^lox/lox\) mutant mice (designated as Olig1:Gnas) generated by breeding Gnas-floxed and Olig1-Cre mice developed anatomically-distinct tumors that were largely restricted between the caudal posterolateral lobe and the dorsal brainstem around the fourth ventricle (Fig. 5f), and contiguous within the rostral brainstem (Fig. 5g). Olig1:Gnas mice developed relatively intact cerebellar structure and morphology, displaying tumor formation around 3 months of age. Approximately 37.4 ± 4.1% of cells in the tumor tissues expressed Ki67 (Fig. 5h), suggesting that the neoplastic cells are highly proliferative. In tumors of Olig1:Gnas mice, we detected extensive expression of the neuronal markers NeuN, Zic1 and Pax6 (Fig. 5i), a hallmark feature of a primitive neuroectodermal tumor-like MB.\(^40\) In contrast, only a few scattered cells in the tumor were positive for an astrocytic marker GFAP (Fig. 5j).

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Anatomically-distinct tumors from different Gnas mutants resemble SHH-associated MB

Although the localization of MBs is distinct between Olig1:Gnas and GFAP:Gnas mutants (Fig. 1), transcriptome profiling by RNA-seq (Fig. 6a) and qRT-PCR (Fig. 6b) analysis revealed a significant up-regulation of Shh pathway components in tumors of either origin. Regression analysis reveals a direct correlation of gene transcription profiles between GFAP:Gnas and Olig1:Gnas tumors (Fig. 6c). Cross-species comparison of gene expression profiles of Gnas tumors with data from human MB subgroups using two different class prediction algorithms AGDEX41 (Fig. 6d,e) and PAM42 (Fig. 6f) revealed that the tumors derived from both GFAP:Gnas and Olig1:Gnas mice showed a gene expression signature most closely resembling the SHH-group human MB (Fig. 6df). Therefore, our data highlight Olig1⁺ progenitors in the posterior fossa as an important source of a subset of Shh-associated MBs with heterogeneous cellular origins3.

To further examine the tumorigenic capacity of neoplastic cells in these Gnas mutants, we transplanted cells isolated from tumor tissues from GFAP:Gnas or Olig1:Gnas mutants by stereotactically injecting them into the forebrain in immuno-compromised nude mice. Tumor cells were able to propagate into the brain in the transplanted animals and became obvious one month after transplantation (Supplementary Fig. 13). Tumors formed in mouse allografts persisted with GNP-like tumor cells exhibiting MB histology (Supplementary Fig. 13). Congruent with gene profiling and histological data, this suggests that the tumors in GFAP:Gnas or Olig1:Gnas mutants comprise transformed neoplastic cells, resembling SHH-driven MBs. Moreover, to identify the subgroup affiliation of MB in the patient with a homozygous GNAS-inactivating mutation 9, we carried out targeted gene-expression profiling analysis and observed a significant upregulation of signature genes in the SHH-group but not in WNT, group 3 or 4 MB (Fig. 6g). Thus, our results predict that the tumor carrying the GNAS nonsense mutation is a SHH-subgroup MB with high confidence (Fig. 6g). Furthermore, a recent genome sequencing study identified eight cases out of 133 SHH-MBs carrying GNAS mutations including one case with a frame-shift mutation 5 (Supplementary Table 1). Together with tumor formation in Gnas mutants, these studies suggest that GNAS inactivating mutations could lead to SHH-MB formation in patients.

Discussion

We demonstrate here that the Gnas-encoded GPCR signal transducer Gsα is a potent tumor suppressor in Shh-driven MBs. Gnas expression determines progenitor cell competency in initiation of MBs among distinct cells of origin. Low levels of GNAS define a subset of aggressive SHH-MB, highlighting GNAS as a prognostic biomarker for treatment stratification of SHH-associated tumors. The case report that the patient with a homozygous GNAS nonsense mutation 9 developed SHH-MB provides additional clinical relevance of GNAS deregulation in tumor formation. Our gain- and loss-of-function studies suggest that Gsα inhibits MB formation at least in part by suppressing Shh signaling through activating the cAMP-dependent pathway to regulate Gli3 processing and Gli2 activation, as well as modulating ciliary trafficking of hedgehog signaling components in GNPs. Thus, a dual-mode regulation of the intracellular cAMP signaling cascade and Smo activation at the primary cilium by Gsα reinforces the inhibition of Shh signaling and blocks MB initiation.
Our data further suggest that Gα might serve as a point of convergence between Smo and various Gα-coupled GPCR signaling pathways to modulate Shh signal strength and control MB formation. We did not detect significant alterations of gene loci encoding hedgehog-pathway components such as *PTCH1*, *SMO* or *SUFU* in GNAS-low SHH-MBs and in Gnas tumors (Supplementary Fig. 14, Table 1), suggesting that GNAS deregulation or inactivation may represent a unique subset of SHH-MBs. Nonetheless, our copy number variation study revealed a number of other genetic alterations occurred in both *GFAP*:Gnas and *Olig1*:Gnas mutants. The altered genetic loci harboring homozygous deletions include Tulp4, a candidate tumor suppressor gene in MB and Hjurp, a critical factor for chromosome segregation and stability (Supplementary Fig. 14). These genetic alterations might potentially contribute to transforming mechanisms in Gnas mutants.

We identify *Olig1* progenitors as a cellular source for Shh-associated tumors localized to the dorsal brainstems, demonstrating the cellular and anatomic heterogeneity within SHH-MB. Recent studies indicate that *Olig1* may regulate the fate switch between OPC and cerebellar interneurons, raising the possibility that interneuron precursors might contribute to MB formation. However, this seems unlikely because Shh-driven MBs are derived from lineage-restricted GNPs even with SmoM2 activation or *PtcH1* mutation in multipotent cerebellar progenitors. Intriguingly, the ventral brainstem of *GFAP*:Gnas mice exhibits an expanded pontine grey nucleus derived from lower rhombic lip progenitors, which could also act as a source of Wnt-subgroup tumor. Thus, Gα may suppress MB formation in different types of progenitor cells. Together, our studies uncover Gnas as a potential molecular link among anatomically distinct MBs, pointing to a previously unrecognized tumor suppressor function of Gnas in the initiation of diverse MBs in light of other tumor types often caused by activating GNAS mutations.

Although Smo inhibitors show efficacy regarding the inhibition of MB growth in animal models such as *PtcH1* mutants and adult Shh-driven MB patients, drug responses were only transient due to the emergence of drug resistance. Our data suggest that the cAMP stimulants such as Rolipram, which possesses anti-tumor activity and is clinically approved already as an antidepressant in humans in Japan and Europe, might be a potent alternative agent against aggressive SHH-MBs caused by GNAS inactivation. Although other cAMP raising agents have been previously proposed for the treatment of human tumors exhibiting overactive Shh pathways, our current spontaneous MB model with Gnas inactivation provides important and novel validation of the efficacy of Rolipram in vivo. This suggests that Rolipram and perhaps other cAMP-raising agents including bioflavonoids, which overcome multi-drug resistance in cancer therapy, might be repurposed for treating MB. The profound and specific impact of Gα on MB tumorigenesis illuminates a new alternative treatment avenue, such that co-targeting of Gα or its signaling effectors together with Smo inhibition might circumvent the drug resistance seen with Smo antagonists alone and could be beneficial in treatment of aggressive SHH-MB with GNAS deregulation.

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Online Methods

Animals

We bred Gnas<sup>lox/lox</sup> mice with hGFAP-Cre (Jackson laboratory), Atoh1-Cre (gift from Dr. Bernd Fritzsch, University of Iowa) and Olig1-Cre mice to generate hGFAP<sup>Cre<sup>+/−</sup>:Gnas<sup>lox/+</sup>, Atoh1<sup>Cre<sup>+/−</sup>:Gnas<sup>lox/+</sup> and Olig1<sup>Cre<sup>+/−</sup>:Gnas<sup>lox/+</sup> mice, respectively, which were then bred with Gnas<sup>lox/lox</sup> mice to produce control mice (hGFAP<sup>Cre<sup>+/−</sup>:Gnas<sup>lox/+</sup>, Atoh1<sup>Cre<sup>+/−</sup>:Gnas<sup>lox/+</sup> and Olig1<sup>Cre<sup>+/−</sup>:Gnas<sup>lox/+</sup>), and Gnas mutant offspring hGFAP<sup>Cre<sup>+/−</sup>:Gnas<sup>lox/lox</sup> (GFAP:Gnas), Atoh1<sup>Cre<sup>+/−</sup>:Gnas<sup>lox/lox</sup> (Atoh1:Gnas) and Olig1<sup>Cre<sup>+/−</sup>:Gnas<sup>lox/lox</sup> (Olig1:Gnas) mice, respectively. The above control mice developed and behaved the same as wildtype. Rosa-tomato reporter mice (Jackson laboratory) and Atoh1-GFP reporter line (gift from Dr. Jane Johnson) were also bred with Gnas mutants to monitor gene deletion and Atoh1-expressing GNP cells, respectively. We used both male and female mice for the study. The mouse strains used in this study were generated and maintained on a mixed C57Bl/6;129Sv;CD-1 background. BALB/c nude mice (Charles River Laboratories) were used for allograft transplantation study. All animal use and studies were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center, Cincinnati Children’s Hospital Medical Center, USA and animal use committee at Sichuan University in Chengdu, China. All human patient samples were obtained with consent as outlined by individual institutional review boards.

Tissue processing, antibodies, immunostaining and microscopy

Mouse brains at defined ages were dissected and fixed overnight in 4% (w/v) paraformaldehyde (PFA) and processed for cryosectioning or paraffin embedding and sectioning. The procedure for immunostaining was described previously. Briefly, for tissue immunostaining, cryosections or pre-deparaffinized tissue sections were incubated overnight in primary antibodies diluted in block solution (PBS with 5% v/v normal goat serum (Sigma-Aldrich) and 0.3% v/v Triton X-100). After washing with PBS for five times, sections were then incubated overnight in appropriate either biotinylated secondary antibodies, followed by using the ABC avidin/biotin method to visualize staining signals under light microscopy with the peroxidase/diaminobenzidine (DAB) method, or incubated with corresponding fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch) under fluorescent microscopy. For cell immunostaining, cultured cells were fixed with 4% PFA for 10 min and washed five times with PBS, then placed in blocking solution for 30 min. We incubated primary antibodies in blocking solution with proper dilutions and stain cells for 1 h at room temperature. For BrdU staining, cells or tissue sections were denatured with 0.1N HCl for 1 h in 37°C water bath. After denaturation, sections were neutralized with 0.1 M Borax, pH 8.5 (Sigma) for 10 min. Sections were washed with 0.3% Triton X-100/1 × PBS (wash buffer) for 3 times and blocked with 5% normal donkey serum (Sigma-Aldrich) contained wash buffer for 1 h at room temperature. Mouse-anti BrdU (BD Bioscience, 1:500) antibody was used to label BrdU overnight at 4°C. DAPI was included in the final washes before the samples were mounted in Fluoromount G (SouthernBiotech) for microscopy. Primary antibodies used in this study were as follows: Zic1 (Rockland, 200-401-159), Olig2 (gift of C. Stiles, Harvard Medical
School), GFAP (Sigma, G3893), Gsα (Santa Cruz, Sc-823), Smoothened (Smo) (LS-C47301, LSBio), acetylated α-tubulin (mAb 6-11B-1, Sigma), Patched1 (gift of Rajat Rohatgi, Stanford University), Gpr161 (gift of Suzie Scales, Genentech, Inc), Ki67 (Thermo Sci, Clone: SP6), Pax6 (Developmental Studies Hybridoma Bank-University of Iowa), Tuj1 (Covance, MMS-435P), BrdU (BD Bioscience 347580), NeuN (Millipore, MAB377), Arl13b (Santa Cruz, sc-102318), Gli2 and Gli3 (gift of Suzie Scales, Genentech, Inc).

For microscopy and image acquisition, images of stained samples used in figures were collected on an inverted laser scanning confocal microscope (Carl Zeiss LSM 510) microscope equipped with high-efficiency fluorochrome specific filter sets for DAPI, Cy2, Cy3 and Cy5. For quantification of the number of Ki67, BrdU or Zic1-expressing cells, areas to be counted were traced with a 40× objective lens and sample frames (40 μm × 40 μm) were selected at least 10 random but non-overlap regions per section by the image analysis software. At least five sections at different hindbrain levels per animal were selected for quantification. Imaging of cilia was taken with a Zeiss immersion oil objective lens (63 ×) with 2× optical zoom magnifications. Experiments were performed at least three times for each genotype. At least 300 cells with visible cilia from each independent experiment per genotype were analyzed. Images were quantified in a double-blinded manner.

RNA in situ hybridization of brain sections was performed using digoxigenin-labeled riboprobes as described previously 17. The probes used were: murine Ptch1, Gli1, Gli2 and Cyclin D1. Detailed protocols are available upon request.

Cerebellar GNP culture and proliferation assays

Cerebella from P6 or P7 mice were digested with Trypsin/DNase (1 mg ml⁻¹; Worthington), triturated to obtain a single-cell suspension, and then centrifuged through a 35%–65% Percoll gradient (Sigma) according to Yang et al., 2008 13. Cells from the 35%–65% interface were suspended in the GNP culture medium [Neurobasal (Gibco) with 2 mM L-glutamine, 0.45% D-glucose, B27 supplement, 16 μg ml⁻¹ N-Acetyl-L-Cysteine and penicillin/streptomycin]. We pre-plated GNPs onto poly-D-lysine (100 μg ml⁻¹) coated plates for 1 hr at 37°C twice, and then transferred them to Poly-D/L-ornithine coated plates for culture. We treated GNP cells from control and Gsα mutants with following agents: SAG (Enzo, Lx-270-426) 200 nM; forskolin (Sigma, F3917) 10 μM; a selective Gas antagonist NF449 (http://www.tocris.com/dispprod.php?ItemId=1801#.UwpK4v3j7Ko) (Tocris, Cat. # 1391) 200 μM; GDC-0449 (Selleckchem, S1082) 1 μM and Rolipram 50 μM (R&D, Cat.# 0905) or transfected the cells with pcDNA3 or pGsCA for 48 hr. For in vitro proliferation assays, we labeled GNP cells with BrdU (50 μg ml⁻¹) for 48 hr followed by immunostaining.

Real-time RT-PCR Analysis

RNAs were isolated with the RNeasy Plus Mini kit (Qiagen) from GNP cells or snap-frozen tumors. Reverse transcription was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). We analyzed each gene with at least two different primer sets. qRT-PCR was carried out using the ABI Prism 7700 Sequence Detector System.
(Perkin-Elmer Applied Biosystems) using Gapdh (TaqMan kit, Applied Biosystems) as an internal control. Each analysis was performed in triplicate, and the results were normalized to Gapdh for each sample. The primer sequences for qRT-PCR are available upon request.

**Western blotting**

GNP cells were rinsed in PBS and lysed in modified RIPA buffer (50 mM Na-Tris, pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.25% sodium deoxycholate, 1 mM diethiothreitol, 10 mM NaF, 1 mM active sodium vanadate, 1 mM PMSF and 1 × a cocktail of cOmplete protease inhibitors (Roche Applied Science) and centrifuged at 13,000 rpm for 15 min at 4 °C. After the determination of protein concentration (Bio-Rad), the lysates were separated by 4–12% SDS-PAGE. We performed western blotting using standard protocols. The antibodies used were as follows: rabbit antibodies to Gli3 for detecting Gli3FL and Gli3R forms (gift of Suzie Scales), Gsα (Santa Cruz, Sc-823), anti-phospho-PKA substrates (P-PKAs, Cell Signaling, 9624s) and GAPDH (Santa Cruz, FL-335). Bands were visualized with secondary antibodies conjugated to horseradish peroxidase (Bio-Rad) and ECL western blotting detection reagents (Pierce) per the manufacturer's instructions. We used ImageJ densitometry software for western blot quantification.

**Assay for activated Gsα (Gsα-GTP)**

We performed activated Gsα-GTP pull-down assays following the manufacturer's protocol (NewEast Bioscience, 80801). Briefly, GNPs were incubated with Shh (3 μg ml<sup>–1</sup>, R & D), XAV-939 (1 μM) and SAG (200 nM) for 1 h. Cell lysates were incubated with the antibody against the active form Gsα-GTP followed by protein A/G agarose beads for 1 h. Activated Gsα and Gsα proteins were detected by western blot with Gsα antibody.

**Enzyme-linked immunoassay (ELISA) for cAMP**

We plated GNP cells on 96-well tissue culture plates and cultured them for 1 day. The cAMP level in the GNPs was assayed following the manufacturer's protocol (Cell Signaling, 4339). In brief, GNPs were treated with SAG (200 nM), NF449 (100 μM) or forskolin (10 μM) for 24 hr. The amount of cAMP in lysates from 8,000 GNP cells was measured by ELISA.

**RNA-seq data analysis**

We isolated RNAs from the cerebella of adult wildtype mice and tumor tissue from individual GFAP:Gnas or Olig1:Gnas mutants and subject to RNA deep sequencing. RNA-seq libraries were prepared using Illumina RNA-seq Preparation Kit (Illumina) and sequenced in HiSeq 2000 sequencer. RNA-seq reads were mapped using TopHat with default settings. TopHat output data was then analyzed by Cufflinks to (1) calculate FPKM values for known transcripts in mouse genome reference and (2) test for changes of gene expression of control and tumor tissues.

**Copy number variation analysis**

We isolated genomic DNAs from the cerebellum of adult wildtype mice and tumor tissue from individual GFAP:Gnas or Olig1:Gnas mutants (n = 3 for each genotype) using a DNA
preparation kit (Zymo Inc.) and were hybridized on aCGH arrays (Nimblegen, Mouse CGH 3x720K Whole-Genome Tiling Array) according to the manufacturer’s protocol. Data from the aCGH arrays were analyzed using the Nexus aCGH software with recommended normalization settings. Each sample was compared to a distributed baseline to identify amplified and deleted regions using a segmentation algorithm within Nexus Suite using a cutoff with gain (≥0.23) and loss (≤–0.5) and significance threshold = 1.0E–5. Segments showing copy number variation were only reported if they occurred in list those regions of gain or loss with an individual False Discovery Rate (FDR) no greater than 5%.

Classification of medulloblastoma patient subgroups

Classification of a group of 103 paraffin-embedded MB samples was established using unsupervised hierarchical clustering (HCL) as the training series described previously.\textsuperscript{57,58} Unsupervised HCL of MB expression data identified the following four unique sample clusters: WNT group, SHH group, group C, and group D. Briefly, gene expression data from MB patient samples were generated by using Affymetrix HTHGU133Achips (Affymetrix, Santa Clara, CA). Molecular subgroups were classified using TM4 Microarray Software Suite (MeV v4.4; Dana-Farber Cancer Institute, Boston, MA). Subgroup-specific signature genes were identified by a multivariate permutation test. We performed principal component analysis (PCA) of gene expression data using Partek Genomics Suite (Partek, St Louis, MO).

Subgroup determination of the tumor with a homozygous GNAS nonsense mutation from a 14-month-old post-mortem infant: Total RNA was extracted from FFPE tumor tissue using the Qiagen RNeasy FFPE Kit (Qiagen, Hilden, Germany) and 200 ng of total RNA were analysed on a nanoString nCounter using a custom 25 gene probeset as previously described.\textsuperscript{58} Counts were normalized to the three housekeeping genes (GAPDH, ACTB and LDHA) and subgroup prediction was done using PAM (Prediction analysis of microarrays) as previously described using the R-statistical environment (v2.15.1).\textsuperscript{58}

Molecular classification of mouse tumors

Mouse tumors and normal cerebella (n = 8 each group) were profiled on the Affymetrix GeneChip Mouse Gene 1.1 ST v1 platform. Transcript level Robust Multi-array Average (RMA) normalization was performed using the oligo package (v 1.14) in the R environment (v 2.15). Mouse transcripts were mapped to Human transcripts using gene orthology predicted by EnsemblCompara GeneTrees\textsuperscript{60} available on Ensembl BioMart (GRCm38 dataset). Subsequently, the expression profiles were analyzed to assign molecular MB subgroup to the mouse tumors, using the AGDEX R package (v 1.0.1) and human MB expression data from Northcott et al.\textsuperscript{57} In this cross-species comparison of global differential expression, mouse normal cerebellum and human normal cerebellum were used as references. The degrees of agreement in differential expression of the mouse tumors between each of the four human MB subgroups were assessed separately and tested for statistical significance using permutation tests.

The molecular classification of the mouse tumors were also performed using a class prediction algorithm, Prediction Analysis for Microarrays (PAM)\textsuperscript{42}, as implemented in the
pamr package (v 1.51). The mouse and human samples first were normalized to their respective cerebellar references. Subgroup-specific markers were identified based on Kruskal-Wallis tests with multiple hypothesis correction by the Benjamin-Hochberg method, using a false discovery rate threshold of 0.01 and a signal-to-noise ratio threshold of 1.5. The resulting 545 subgroup-specific signature markers were used as features for class prediction of the mouse tumors using a PAM classifier trained on the human MB samples. Predicted subgroups with confidence probabilities higher than established thresholds were considered bona fide subgroup assignments. Plots were generated by Principal Component Analyses (PCA) on the expression profiles of human medulloblastomas (training data). The resulting eigenvectors were used to project the expression profiles of the classified samples onto the vector space spanned by the first two eigenvectors of the training data. The background confidence score gradient was generated using 200 replicates of the training data with added Gaussian noise and subsequently smoothed by Nadaraya-Watson normalization (fields v6.7.6 R package).

**Rolipram treatment in mice and volumetric measurement of tumors**

Control and GFAP:Gnas mutant mice at P30-P35 were randomized to receive either Rolipram (5 mg kg$^{-1}$) or vehicle control [5% (v/v) DMSO] administered twice daily via intraperitoneal injection as previously described. Brain tissues were harvested and processed into 8-μm sections in the sagittal plane and stained with hematoxylin and eosin (H/E) or subject to immunostaining. Abnormal tissue area with densely packed cells and round-to-oval hyperchromatic nuclei was assumed to be tumor tissue in the tissue section. For volumetric measurement, sections of cerebella were scanned by using a ScanScope XT from Aperio (Vista, CA) to acquire serial section images, then stacked and aligned with the StackReg function of ImageJ to generate three-dimension models and analyzed with Imaris Software (Bitplane) to calculate the volume of tumor tissues.

**Intracranial transplantation**

Tumor cells isolated from Gsα mutants were plated in GNP culture medium, and harvested with 0.25% trypsin and 0.02% EDTA for 2 minutes, washed twice with Hank’s balanced salt solution (HBSS), and resuspended in Ca$^{2+}$ and Mg$^{2+}$-free HBSS. Cell viability was determined by trypan blue exclusion. Only single-cell suspensions with more than 90% viability were used for in vivo allograft studies. Cells ($5 \times 10^{5}$) were stereotactically injected into the lateral ventricle of nude mice (6-week-old BALB/c nu/nu; coordinates: anterior–posterior, +1.8; medial–lateral, +2.2; dorsal–ventral, -2.0 mm from Bregma).

**Statistical analysis**

All analyses were done using Microsoft Excel or Prism GraphPad 6.00 for Mac OS (San Diego California, www.graphpad.com). Quantifications were performed from at least three independent experimental groups. Data are presented as mean ± S.E.M. in the graphs. p-values are from Student’s two-tailed t test to compare two sets of data. To compare more than two sets, one-way analysis of variance analysis (ANOVA) with a Newman–Keuls multiple comparison test for post-hoc analysis. Survival analyses used animal death times and censoring times when animals were sacrificed or as otherwise stated. Survival curves
were plotted with the Kaplan–Meier method and compared by using a two-sided log-rank test. In human tumor data analysis, Fisher’s exact test was used for data in Tumorscape database unless otherwise specified. $p < 0.05$ is considered to be statistically significant.

** Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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** References**


GNAS defines a subset of aggressive SHH-group tumors
(a-d) MB patients with available survival information and gene expression profiling studies from both Boston and Heidelberg series of MBs were divided into two groups using the median GNAS expression value as the cutoff point. The relationship between GNAS mRNA expression and survival time was analyzed according to the Kaplan-Meier method, using log rank statistics. GNAS levels and patient numbers: a, low (n =16), high (n =17); b, low (n = 10), high (n = 10); c, low (n = 32), high (n = 32); d, low (n = 64), high (n = 65).
Figure 2. Loss of Gnas in neural stem/progenitor cells induces MB formation
(a) Sagittal brain sections from hGFAPCre::Gnas<sup>lox/lox</sup> (GFAP::Gnas) and hGFAPCre::Gnas<sup>lox/+</sup> (Ctrl) mice at indicated stages were stained with hematoxylin and eosin (H/E).

(b) Brain appearance of control and Gnas mutants at P67. The arrows indicate the cerebellum.

(c) Tumors from Gnas mutants (left) displays similar histology to human MB (right; SHH group). Insets are shown at high magnification.

(d) The cerebella of control and Gnas mutants at P60 were stained with anti-Ki67 and DAPI.

(e) Kaplan-Meier survival curves for control and GFAP::Gnas mice (n = 52).

(f) Heatmap shows expression of Shh pathway components in control cerebella and GFAP::Gnas tumor tissues. The color bar shows expression intensity.

(g) qRT-PCR quantification of Gnas and Shh pathway genes in control and GFAP::Gnas cerebella at P30. Data represent the mean ± SEM (n = six animals). ** P < 0.01; Student’s t test.

(h) mRNA expression of Shh target genes as indicated in control and GFAP::Gnas brain sections at P60. Arrow and arrowhead indicate the cerebellum and pontine grey nucleus, respectively.

(i) qRT-PCR analysis of Gnas, Gli1 and Ptc1 in GNPs from control and GFAP::Gnas mice at P7. Data represent the mean ± SEM (n = five animals). ** P < 0.01; Student's t test.

(j) The cerebellar EGL region of GFAP::Gnas mice carrying the Atoh1-GFP reporter at P50 was immunostained with anti-Zic1, Olig2 and GFAP as indicated.
Scale bars in a, 300 μm; b, 5 mm; c, d, h, 200 μm; inset in c, 10 μm; j, 100 μm.
Figure 3. Gsα and its effector cAMP inhibit Shh signaling and tumor growth in Gnas mutants

(a) Bar graph (left) depicts expression changes in GNPs treated NF449 over control. Right: cAMP levels measured by ELISA.

(b) GNPs were treated with vehicle and SAG (200 nM) and/or transfected with pcDNA3 control and GsαQ227L (pGsCA) for 48 h. Bar graphs depict Gli1 and Ptc1 expression changes in treated cells over control.

(c) Average cAMP levels of control and mutant GNPs treated with vehicle and forskolin.

(d) GNPs were treated with vehicle or Rolipram (50 μM), forskolin (10 μM) and db-cAMP (100 μM) for 3 h. Gli1 and Ptc1 or Gli3FL and Gli3R were analyzed by qRT-PCR (left) and western blotting (right) as indicated. GAPDH: loading control.

(e) Quantification of Gli1, Ptc1 and Ccnd1 in GNPs treated with H89 or KT5720 over control.

(f) Bar graphs depict the relative fold change of Gli1 and Ptc1 expression in GNPs transfected with pGsCA with or without H89 treatment over control.

(g) Control and Gnas mice (n = eight for each group) were randomized to receive Rolipram or vehicle from P35 to P65. Representative images of H/E staining of cerebellar sections and brain morphology were shown. Arrows: the cerebellum.

(h) Scatter dot-plot depicts average tumor volumes estimated for vehicle- and Rolipram-treated Gnas mutants. Lines: mean values ± SEM.

(i) Images show immunostaining of Zic1 and BrdU in vehicle and Rolipram-treated GFAP:Gnas tumors at P65. Insets: high magnification in boxed areas. Bar graph (right) depicts the percentage of BrdU+/Zic1+ cells (n = eight animals each group).
(j) Kaplan-Meier survival curves for GFAP:Gnas mice (n = 12 per group) were randomized to receive Rolipram or vehicle at P30 for six weeks. P value for the log-rank test =0.0071. Scale bars in g, 300 μm; i, 20 μm (insets 4 μm). Data shown in a-f are the mean ± SEM representing at least three independent experiments. *P < 0.05, **P < 0.01; Student’s t-test.
Figure 4. Gsα regulates ciliary trafficking of hedgehog signaling components in GNPs

(a-b) GNPs without or with Shh treatment (3 μg ml⁻¹) for 16 hr were immunostained with anti-Gsα and acetylated α-tubulin (Ac-Tub) (arrows). b: quantitation of Gsα fluorescence at the primary cilium (≥300 cell counts each group per experiment).

(c-g) GNPs from control or GFAP:Gnas mice were immunostained with anti-Gli2, Smo, Ptch1 and Ac-Tub. Insets in c show cilia at a high magnification. Bar graphs in d, g depict the percentage of Gli2 accumulation at cilium tips, and Smo or Ptch1 fluorescence at the primary cilium, respectively (≥300 cell counts per genotype from each experiment). Arrows and arrowheads indicate primary cilia and their base, respectively.

(h) GNPs from Gnas mutants were treated with GDC-0449 (1 μm) or both GDC-0449 and Rolipram (50 μm). Bar graphs depict relative Gli1 and Ptch1 expression by qRT-PCR in drug-treated vs. vehicle-treated cells.

(i) Zic1 and BrdU immunostaining in GNPs from Gnas mutants treated GDC-0449, Rolipram or both and labeled with BrdU for 48 hr.

(j) Bar graph depicts the average percentage of BrdU⁺ cells among Zic1⁺ GNPs. Experiments were performed three times with at least n = 3 for each treatment. One-way ANOVA with post hoc Newman–Keuls multiple comparison test. **P < 0.01.

Scale bars in a-f, 3 μm; c insets, 0.5 μm; i, 30 μm. Representative images and quantifications from at least three independent experiments are shown. Data represent the mean ± SEM. *P < 0.05, **P < 0.01, Student’s t-test or one-way ANOVA.
Figure 5. Loss of Gnas in Atoh1⁺ or Olig1⁺ progenitors leads to an anatomically distinct Shh-associated MB

(a) A sagittal hindbrain section from a Atoh1:Gnas mouse at P50 was stained with H/E. The boxed area is shown at a high magnification in the right panel.
(b) Tumor tissues were immunostained with anti-Tuj1, Olig2, Zic1 and GFAP as indicated.
(c) Bar graphs depict expression of Ptc1, Gli1 and Hhip in Atoh1:Gnas cerebella over control at P40. Data represent the mean ± SEM from five animals each group. ** P < 0.01; Student’s t test.
(d) Olig1 expression (arrow) was detected in the progenitors of the dorsal brainstem at sagittal levels at E15.5 by in situ hybridization.
(e) The dorsal brainstem region from Olig1-Cre:Rosa-tdTomato mice at P7 was immunostained with Zic1. Arrows indicates a population of tdTomato⁺ cells Zic1.
(f-g) H/E staining of the sagittal sections of Olig1:Gnas brains at 3 or 5 month ages. Arrows indicate the tumor tissue. Boxed region in f is shown at high magnification in g. BS: brainstem.
(h-j) Sections of Olig1:Gnas tumor tissues were immunostained with anti-Ki67, NeuN, Zic1, Pax6 and GFAP as indicated.
Scale bars in a, 50 μm. b, 20 μm, d, f, 200 μm; e, 20 μm; g-j, 50 μm.
Figure 6. Tumors in Gnas mutants exhibit a gene expression signature resembling SHH-MB

(a) Heatmap analysis of gene profiling of control cerebella (n = 3) and tumor tissues (n = 8) from Olig1:Gnas mutants by RNA-sequencing shows upregulation of Shh pathway components in tumors. The color bar shows expression intensity.

(b) Relative expression of Shh pathway components between control and Olig1:Gnas tumors from four month old animals (n = eight per group) was assayed by qRT-PCR. ** p < 0.01; Student's t test.

(c) Regression analysis of gene expression profiles indicates a direct correlation of gene transcription profiles between GFAP:Gnas and Olig1:Gnas tumors (n = eight per group).

(d-e) Cross-species comparison of global differential expression from Affymetrix microarray analysis of mouse tumors (n = eight per group) with bona fide human MB subgroups by AGDEX3 R algorithm. Bar graphs represent the cosine similarity measure and reflect the similarity of global expression profile between each mouse tumor subtype and each human MB subgroup.

(f) Principal component analysis (PCA) of expression profiles between human and above mouse tumor samples. Arrows indicate that gene expression profiles of mouse tumors match to Shh-subgroup.

(g) Subgrouping analysis by nanoString technology indicates the MB from the patient with a GNAS homozygous nonsense mutation resembles a SHH-group tumor with high confidence (PAM prediction score = 0.999996).