The G protein α subunit Goαs is a tumor suppressor in Sonic hedgehog–driven medulloblastoma

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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 Goαs, as a potent tumor suppressor gene that, when expressed at low levels, defines a subset of aggressive Sonic hedgehog (SHH)-driven human medulloblastomas. Ablation of the single Gnas gene in anatomically distinct progenitors in mice is sufficient to induce Shh-associated medulloblastomas, which recapitulate their human counterparts. Goαs is highly enriched at the primary ciliary of granule neuron precursors and suppresses Shh signaling by regulating both the cAMP-dependent pathway and ciliary trafficking of Hedgehog pathway components. Elevation in levels of a Goαs effector, cAMP, effectively inhibits tumor cell proliferation and progression in Gnas-ablated mice. Thus, our gain- and loss-of-function studies identify a previously unrecognized tumor suppressor function for Goαs that can be found consistently across Shh-group medulloblastomas of disparate cellular and anatomical origins, highlighting G protein modulation as a potential therapeutic avenue.

Medulloblastoma (MB) comprises clinically and molecularly distinct tumors that arise either in the cerebellum or brainstem1–3. 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 medulloblastoma4–6; however, the underlying signal transduction events that drive tumor initiation and progression remain obscure. GNAS encodes the heterotrimeric Gs protein α subunit (Goαs), which functions as a molecular switch to transmit various GPCR signals to control cell growth, survival and motility7. Recent genome-wide analyses of somatic mutations in cancers identified GNAS as one of the most frequently mutated genes8. Although most somatic tumor types acquire gain-of-function GNAS mutations9, our analysis of a copy number database (Tumorscape, Broad Institute) unexpectedly revealed that MB displays a marked 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 previously unknown homozygous nonsense mutation within the GNAS coding region developed MB9. Herein, we tested whether deregulation of Goαs-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, WNT (Wingless) group, SHH group, group 3 and group 4, on the basis of their distinct gene expression profiles10. To determine the correlation of GNAS in MB subgroups, we examined GNAS expression from two independent, nonoverlapping patient cohorts in Boston and Heidelberg10–12. We found that low GNAS expression tightly correlated with significantly decreased overall survival within SHH-group tumors (SHH-MB), which comprise approximately 30% of all MBs1. Notably, we did not observe the prognostic impact of low GNAS expression in other group tumors and across MB subgroups (Fig. 1c,d and Supplementary Figs. 2 and 3). These observations

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suggest that low expression or loss of GNAS specifically defines a subset of aggressive SHH-group MBs.

Loss of Gnas in neural progenitors induces MB formation

To determine whether Gnas inactivation could lead to brain tumorigenesis, we deleted Gnas in neural stem/progenitor cells by breeding mice with floxed Gnas (Gnasfl/fl) with a human GFAP promoter–driven Cre (hGFAP-Cre) mouse line13,14. Strikingly, all resulting hGFAP-Cre–hemizygous Gnasfl/fl conditional-knockout mice (designated as GFAP-Gnas mice) 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. GFAP-Gnas 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), resembling the histological features of human MB15. In the mutants at postnatal day 60 (P60), the neoplastic cells were highly proliferative, as indicated by extensive expression of Ki67,
which was barely detectable in Gnas<sup>Bl+</sup> control mice (Fig. 2d). 100% of Gnap-Gnas animals succumbed to their tumor around 3–4 months of age (Fig. 2e). Although the hGAP-Cre—recombined cells appear in most brain regions<sup>13</sup>, tumor formation was confined to the cerebellum during the lifespan of Gnap-Gnas mice.

To ascertain gene expression alterations caused by Gnas loss, we examined mRNAs isolated from the cerebella of control and Gnap-Gnas mice at P60 by RNA deep sequencing. In tumors of Gnap-Gnas mice, our data revealed an upregulation of Shh signaling pathway components (Fig. 2f). Quantitative RT-PCR confirmed that expression of Shh target genes and pathway components was significantly upregulated (Fig. 2g). Consistent with this finding, mRNA in situ hybridization revealed intense expression of Shh-downstream genes including Gli1, Gli2, Pch1 and Cnd1 (encoding Cyclin D1) (Fig. 2h). Furthermore, we observed significant elevation of Shh direct target genes Gli1 and Pch1 (Fig. 2i) in GNP-like tumor cells compared with normal GNPcs, suggesting a cell-intrinsic effect of Gnas mutation on Shh signaling activation. By contrast, expression of Wnt-target genes was not substantially altered (Supplementary Fig. 4), which is consistent with previous findings that Gnas loss does not affect Wnt signaling in other cellular systems<sup>16</sup>. We observed widespread expression of the GNP markers Zic1 and Atoh1 (also known as Math1), along with Shh-regulated targets, including Olig2 (ref. 17), but very few astrocytic GFAP<sup>+</sup> astrocytes (Fig. 2j). Thus, Gnas loss results in an increase or, alternatively, a derepression of physiological levels of Shh pathway activity and over-proliferation of GNP-like tumor cells.

G<sub>Gα</sub><sub>q</sub> activity and cAMP suppress hedgehog signaling and MB growth

To test whether the GTPase activity of Gα<sub>q</sub> protein is required for inhibition of Shh signaling, we treated GNPcs isolated from wild-type neonates with NF449, a selective G<sub>Gα</sub><sub>q</sub> antagonist<sup>18</sup>, which prevents GTP binding to G<sub>q</sub>, and blocks G<sub>Gα</sub><sub>q</sub> GTPase activity. Treatment with NF449 resulted in a significant upregulation of the Shh target genes Gli1, Gli2, Pch1 and Mycn and caused a decrease of intracellular cAMP levels (Fig. 3a), suggesting that inhibition of Gα<sub>q</sub> GTPase activity activates Shh signaling. To investigate the effect of Gα<sub>q</sub> gain-of-function activity on Shh signaling, we generated a constitutively activated form of Gα<sub>q</sub>, Gα<sub>q</sub> Q227L (GsCA), which resulted in an active GTPase-defective, GTP-bound Gα<sub>q</sub> protein<sup>19</sup>. Overexpression of GsCA in GNPcs suppressed the upregulation of Gli1, Pch1, Mycn and Cnd1 induced by the Shh agonist (SAG) (Fig. 3b), indicating that Gα<sub>q</sub> activation inhibits hedgehog signaling.

The classic signal transduction pathway of Gα<sub>q</sub> is through activation of adenylyl cyclase, which, in turn, produces intracellular cAMP<sup>20</sup>, cAMP has been shown to activate cAMP-dependent protein kinase A (PKA), a negative effector of Shh signaling<sup>21–23</sup>. Tumor cells isolated...
Figure 4  Gsα regulates ciliary trafficking of hedgehog components and GNP proliferation. (a) Immunostaining of GNPs without or with Shh treatment (3 µg ml⁻¹) for 16 h with anti-Gαs and anti-α-catenin (Ac-Tub) (arrows). (b) Quantitation of Gαs fluorescence at the primary cilium (≥300 cell counts per experiment per animal, n = three animals, Student’s t-test). (c-g) GNPs from control or GFAP-Gnas mice were immunostained with anti-Gli2 and anti-Ar13b (ADP-ribosylation factor-like protein 13b) to label primary cilia, (c), anti-Smo (e), anti-Ptch1 (f) and anti-Ac-Tub (e,f). Insets in c show cilia at a high magnification. Bar graphs in d and g depict the percentage of Gli2 accumulation at cilia tips, and Smo or Ptch1 fluorescence at the primary cilium, respectively (≥300 cell counts per genotype from each experiment, n = three experiments, Student’s t-test). Arrows and arrowheads indicate primary cilia and their base, respectively. (h) GNPs from GFAP-Gnas mice 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 versus vehicle-treated cells. n = four treatments, Student’s t-test. (i) Zic1 and BrdU immunostaining in GNPs from Gnas mutants treated GDC-0449, Rolipram or both and labeled with BrdU for 48 h. (j) Bar graph depicts the average percentage of BrdU⁺ cells among Zic1⁺ GNPs. n = four treatments. One-way analysis of variance (ANOVA) with post hoc Newman-Keuls multiple comparison test. Scale bars: a, 3 µm; c insets, 0.5 µm; f, 30 µm. Data represent the mean ± s.e.m. *P < 0.05, **P < 0.01.

from GFAP-Gnas mice had a significant reduction in intracellular cAMP levels, whereas treatment of tumor cells with the adenylly cyclase agonist forskolin (FSK) elevated cAMP levels (Fig. 3c). To test the hypothesis that cAMP elevation could inhibit Shh signaling activation, we treated GFAP-Gnas GNPs with rolipram, which elevates cAMP levels by selectively inhibiting phosphodiesterase-4 activity to block cAMP degradation. FSK or a nonhydrolyzable cAMP analog, db-cAMP. Each of these cAMP-raising agents significantly reduced expression of Gli1 and Ptch1 (Fig. 3d). In addition, in wild-type GNPs, treatment with FSK or 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, Ptch1 and Ccnd1 (Fig. 3e). Furthermore, inhibition of PKA activity by H89 (Supplementary Fig. 5) could restore Shh target expression suppressed by constitutively activated Gαs (Fig. 3f). Thus, our data are in keeping with previous observations that Gαs-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. Wild-type control and GFAP-Gnas mice at the young-adult stage, P35, were randomized to receive either vehicle or rolipram intraperitoneally daily for 30 d at a dosage exhibiting effective anti-tumor activity and assessed for tumor development. Rolipram administration during this period did not affect overall cerebellar structure and myelin formation (Fig. 3g and Supplementary Fig. 6). Vehicle-treated GFAP-Gnas mice displayed extensive tumor cell expansion and bulged cerebella (Fig. 3g). In contrast, in rolipram-treated GFAP-Gnas mice, the tumor size and proliferation of Zic1⁺ GNP-like tumor cells were substantially reduced (Fig. 3g-i). In addition, rolipram treatment resulted in a significantly extended lifespan of GFAP-Gnas mice (Fig. 3j). Thus, elevation of the Gαs effector CAMP by rolipram could lead to inhibition of tumor growth in GFAP-Gnas mice.
**Gnas controls ciliary trafficking of Shh signaling components**

Ciliary trafficking of signaling components has an important role in regulating Shh pathway activity and MB formation. Notably, we observed that Gαs was highly enriched at the primary cilium of GNPs (Fig. 4a) but hardly detectable in mouse embryonic fibroblasts (Supplementary Fig. 7), suggesting a unique ciliary function of Gαs for GNP development. In the presence of Shh, ciliary localization of Gαs was diminished (Fig. 4a,b). Treatment of GNPs with Shh or its agonist SAG13 did not alter the total amount of Gαs protein in cells but rather reduced the overall amount of the GTP-bound, active form of Gαs, protein (Gαs-GTP; Supplementary Fig. 8).

In GNPs from GFAP-Gnas mice, Gαs expression was essentially depleted, whereas total levels of Smoothened (Smo) protein were comparable to those in control GNPs (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), whereas Gli2 was hardly detectable or weakly present in wild-type control GNPs (Fig. 4d). Consistent with a principal function of PKA in restraining Gli2 activation21,23, 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 the Smo seven-transmembrane-domain protein in the majority of GFAP-Gnas GNPs, 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 GFAP-Gnas mice (Fig. 4f,g). In contrast, ciliary trafficking of a Shh signaling regulator, Gpr161 (ref. 29), was not altered in GFAP-Gnas GNPs (Supplementary Fig. 10), suggesting a specificity of Gαs in regulating ciliary localization of GPCRs. Consistent with the existence of multiple Gαs-mediated GPCRs, Gpr161−/− embryos exhibited milder developmental defects than Gnas−/− embryos28. Given that PKA null mutation has been shown to lead to Gli2 accumulation in cells but not to ciliary trafficking of Smo in mouse neural progenitors21, our observations of both a strong Gli2 signal at the ciliary tip and Smo ciliary translocation in GFAP-Gnas mice suggest that Gαs might have an additional role in regulating Smo trafficking in primary cilia independent of cAMP-dependent PKA activity.

**cAMP augments Smo inhibition to suppress GNP proliferation**

Our data indicate that Gαs suppresses Shh signaling not only by stimulating intracellular cAMP levels to activate PKA but also perhaps independently by inhibiting Smo translocation. Treatment of GFAP-Gnas GNPs with GDC-0449, which blocks Smo activation induced by SAG (Supplementary Fig. 11)34, led to a reduction in Gli1 and Ptc1 expression (Fig. 4h). Combined treatment with GDC-0449 and rolipram, however, resulted in further inhibition of GFAP-Gnas GNP proliferation than either drug alone (Fig. 4h).

As GFAP-Gnas GNPs are highly proliferative (Fig. 4i), we then determined whether cell proliferation is responsive to cAMP elevation and Smo inhibition. The proliferation rate was modestly reduced in GFAP-Gnas GNPs treated with rolipram or GDC-0449, and rolipram exhibited a relatively stronger effect than GDC-0449 (Fig. 4i,j). However, combinatorial treatment with both drugs caused a greater inhibition of GFAP-Gnas GNP proliferation than either drug alone (Fig. 4i,j).

**Gnas loss in distinct progenitors leads to MB formation**

SHH-driven MBs may arise from multiple cellular origins in human patients. 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 Gnas−/− mice with an Atoh1-Cre line, which directs Cre expression in GNPs of the cerebellum and dorsal brainstem cochlear nuclei3,35. The resulting Atoh1-Cre–hemizygous Gnas−/− (Atoh1-Gnas) mice developed MB-like tumors with an expansion of tumor cells in the EGL (Fig. 5a). Tumor cells expressed the neuronal markers Tuj1 and Zic1 extensively, with few Olig2- and GFAP-expressing glial cells (Fig. 5b), and exhibited a significant upregulation of the 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 progenitors expressing the Shh-regulated gene Olig1 (refs. 36,37). During embryogenesis, Olig1 is mainly expressed in the specified progenitors of the caudal brainstem around the fourth ventricle (Fig. 5d), 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 the astrocytic marker GFAP (Fig. 5j).

Anatomically distinct Gnas-mutant tumors resemble SHH-MB

Although the localization of MBs is distinct between Olig1-Gnas and GFAP-Gnas mice (Fig. 1), transcriptome profiling by RNA-seq (Fig. 6a) and quantitative RT-PCR (qRT-PCR) (Fig. 6b) analysis revealed a substantial upregulation of Shh pathway components in tumors of either origin. Regression analysis revealed a direct correlation of gene transcription profiles between GFAP-Gnas and Olig1-Gnas tumors (n = 8 per group). FPKM, fragments per kilobase of exon per million fragments mapped. (d,e) Cross-species comparison of global differential expression from Affymetrix microarray analysis of mouse tumors from GFAP-Gnas (d) and Olig1-Gnas (e) mice (n = 8 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 the tumor subtype of GFAP-Gnas (d) or Olig1-Gnas (e) mice and each human MB subgroup. (f) Principal component analysis (PCA) of expression profiles between human and GFAP-Gnas or Olig1-Gnas mouse tumors. 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 homozygous nonsense GNAS mutation resembles a SHH-group tumor with high confidence (PAM prediction score = 0.999996).

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 mice by RNA-seq. The color bar shows expression intensity. (b) Relative expression of Shh pathway components between control cerebella and Olig1-Gnas tumors from 4-month-old animals (n = 8 per group) assayed by qRT-PCR. (c) Regression analysis of gene expression profiles showing a direct correlation of gene transcription profiles between GFAP-Gnas and Olig1-Gnas tumors (n = 8 per group). FPKM, fragments per kilobase of exon per million fragments mapped. (d,e) Cross-species comparison of global differential expression from Affymetrix microarray analysis of mouse tumors from GFAP-Gnas (d) and Olig1-Gnas (e) mice (n = 8 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 the tumor subtype of GFAP-Gnas (d) or Olig1-Gnas (e) mice and each human MB subgroup. (f) Principal component analysis (PCA) of expression profiles between human and GFAP-Gnas or Olig1-Gnas mouse tumors. 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 homozygous nonsense GNAS mutation resembles a SHH-group tumor with high confidence (PAM prediction score = 0.999996).
microarrays (PAM) (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. 6d–f). Therefore, our data highlight Olig1+ progenitors in the mouse posterior fossa as a key source of a subset of Shh-associated tumors, consistent with their heterogeneous cellular origins.

To further examine the tumorigenic capacity of neoplastic cells in these Gnas-mutant mice, we transplanted cells isolated from tumor tissues from GFAP-Gnas or Olig1-Gnas mice by stereotactically injecting them into the forebrain of immunocompromised nude mice. Tumor cells were able to propagate in the brain of the transplanted animals, and tumors became obvious upon visual inspection 1 month after transplantation (Supplementary Fig. 13). Tumors formed in mouse allografts comprised 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 mice consist of neoplastic cells, resembling SHH-MBs. Moreover, to identify the subgroup affiliation of MB in the patient with a homozygous GNAS-inactivating mutation, we carried out targeted gene-expression profiling analysis and observed a significant upregulation of SHH-group signature genes but not of WNT-group, group-3 or group-4 MB genes (Fig. 6g). Thus, our results predict that the tumor carrying the GNAS nonsense mutation is an SHH-MB with high confidence (Fig. 6g). Furthermore, a recent genome sequencing study identified 8 cases out of 133 SHH-MBs carrying GNAS mutations, including one case with a frameshift mutation (Supplementary Table 1). These studies suggest that GNAS-inactivating mutations may lead to SHH-MB formation in patients.

DISCUSSION

We demonstrate here that the Gnas-encoded GPCR signal transducer Gαs is a potent tumor suppressor in Shh-MBs. Gnas expression determines progenitor cell competency in initiation of MBs among distinct cells of origin in mice. In humans, we found that low levels of GNAS define a subset of aggressive SHH-MB, suggesting that GNAS should be tested as a prognostic biomarker for treatment stratification of SHH-associated tumors. The case report that a patient with a frameshift mutation most closely resembling the SHH-group human MB provided a Gαs inhibitory oncogene signature. Our gain- and loss-of-function studies suggest that Gαs inhibits MB formation at least in part by suppressing Shh signaling through the CAMP-dependent pathway to regulate Gli3 processing and Gli2 activation, as well as modulating ciliary trafficking of hedgehog-signaling components in GNP. Thus, a dual-mode regulation of the intracellular CAMP signaling cascade and Smo activation at the primary cilium by Gαs reinforces the inhibition of Shh signaling and blocks MB initiation. Our data further suggest that Gαs might serve as a point of convergence between Smo and various Gαs-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, including PTCH1, SMO and SUFU, in GNAS-low SHH-MBs or in Gnas tumors (Supplementary Fig. 14 and Supplementary Table 1), suggesting that MBs with GNAS deregulation or inactivation may represent a unique subset of SHH-MBs. Nonetheless, our copy number variation study revealed that a number of other genetic alterations occurred in both GFAP-Gnas and Olig1-Gnas mice. The altered genetic loci harboring homozygous deletions included Tulp4, a candidate tumor suppressor gene in MBs, and Hjarp, 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 in mice localized to the dorsal brainstem, demonstrating the cellular and anatomic heterogeneity within SHH-MB2,3,5,47. As recent studies indicate that Olig1 may be an arbiter of the oligodendrocyte precursor and cerebellar interneuron fate decision48, it is possible that interneuron precursors might contribute to MB formation. However, this seems unlikely because Shh-driven MBs are derived from lineage-restricted GNP even with SmoM2 activation or Ptc1 mutation in cerebellar stem/progenitor cells13,35.

The ventral brainstem of GFAP-Gnas mice exhibits an expanded pontine gray nucleus derived from lower rhombic lip progenitors, which could also act as a source of Wnt-subgroup tumor39. Thus, Gαs may suppress MB formation in different types of progenitor cells. Together, our studies uncover Gαs as a potential molecular link among anatomic distinct MBs, pointing to a previously unrecognized tumor suppressor function of Gnas in the initiation of diverse MBs, in contrast to other tumor types caused by activating GNAS mutations.8,49.

Although Smo inhibitors efficiently suppress MB growth in animal models such as Ptc1 loss-of-function mutant mice50 and in adult humans with SHH-MB51, in these studies the drug responses were often only transient owing to the emergence of drug resistance. Our data suggest that CAMP stimulants such as rolipram, which possesses anti-tumor activity and is clinically approved already as an antidepressant in humans in Japan and Europe52, might be a potent alternative agent against aggressive SHH-MB caused by GNAS inactivation. Although other CAMP-raising agents have been previously proposed for the treatment of human tumors exhibiting overactive SHH pathways52, our current model of spontaneous MB in GFAP-Gnas mice gives evidence for the efficacy of rolipram in vivo, for which anti-tumor effects had remained unexplored. This suggests that rolipram and perhaps other CAMP-raising agents, including bioflavonoids, which overcome multidrug resistance in cancer therapy53,54, might be repurposed for treating MB. The specific impact of Gαs on MB tumorigenesis suggests a new alternative treatment avenue, such that co-targeting of Gαs or its signaling effectors together with Smo inhibition might circumvent the drug resistance seen with Smo antagonists alone51,55 and could be beneficial in treatment of aggressive SHH-MB with GNAS deregulation.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The RNA-seq, mRNA Affymetrix GeneChip microarray and aCGH microarray data are deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE53248.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
X.H. and Q.R.L. designed the experiments, analyzed the data and wrote the manuscript with input from all authors. X.H., L.Z., Y.C., M.R., D.S., E.L., H.W., Y.Y., Y.D., V.R., X.W. and T.H. carried out the in vitro, in vivo, gene profiling or in situ studies. M.K. and S.M.P. provided whole-genome sequencing data. Y.H., F.W. and W.Z. provided resources and supervision. L.S.W. provided the Gnas floxed mice. D.K.B. and S.H.K. diagnosed and confirmed the Gnas-mutated MB. S.L.P., R.J.G., J.B.R. and W.R.W. provided conceptual advice and edited the manuscript. M.D.T. and Q.R.L. supervised the project.

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The authors declare no competing financial interests.

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AUTHOR CONTRIBUTIONS
X.H. and Q.R.L. designed the experiments, analyzed the data and wrote the manuscript with input from all authors. X.H., L.Z., Y.C., M.R., D.S., E.L., H.W., Y.Y., Y.D., V.R., X.W. and T.H. carried out the in vitro, in vivo, gene profiling or in situ studies. M.K. and S.M.P. provided whole-genome sequencing data. Y.H., F.W. and W.Z. provided resources and supervision. L.S.W. provided the Gnas floxed mice. D.K.B. and S.H.K. diagnosed and confirmed the Gnas-mutated MB. S.L.P., R.J.G., J.B.R. and W.R.W. provided conceptual advice and edited the manuscript. M.D.T. and Q.R.L. supervised the project.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Animals. We bred Gnas<sup>fl/fl</sup> mice<sup>e6</sup> with hGFAP-Cre (Jackson Laboratory), Atoh1-Cre (gift from B. Fritzsch, University of Iowa) and Olig1-Cre<sup>36</sup> mice to generate hGFAP-Cre<sup>e7</sup>; Gnas<sup>fl+/−</sup>, Atoh1-Cre<sup>e7</sup>; Gnas<sup>fl+/−</sup> and Olig1-Cre<sup>e7</sup>; Gnas<sup>fl+/−</sup> mice, respectively. The above control mice developed and behaved the same as wild-type mice. Rosa-tdTomato reporter mice (Jackson Laboratory) and Gnas<sup>fl+/−</sup> mice, respectively. The above control mice developed and behaved the same as wild-type mice. Rosa-tdTomato reporter mice (Jackson Laboratory) and Atoh1-GFP reporter line (gift from J. Johnson) were also bred with Gnas<sup>fl+/−</sup> mice 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.

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<sup>37</sup>. Briefly, for tissue immunostaining, cryosections or pre-deparaformalized 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 either incubated overnight in appropriate 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 appropriate dilutions and stained 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) 3 times and blocked with 5% normal donkey serum (Sigma-Aldrich) in 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, 1:400), Olig2 (gift from C. Stiles, Harvard Medical School, 1:1,000), GEAP (Sigma, G3893, 1:400), Gt3 (Santa Cruz, Sc-823, 1:100), Smootherned (Smo) (LS-C47301, LSBio, 1:500), acetylated α-tubulin (mAb 6-11B-1, Sigma, 1:500), Patched1 (gift from R. Rohatgi, Stanford University, 1:2,000), Gpr161 (gift from S. Scales, Genentech, 1:200), Ki-67 (Thermo Sci, clone Sp6, 1:250), Pax6 (Developmental Studies Hybridoma Bank-University of Iowa, 1:100), Tuj1 (Covance, MMS-435P, 1:100), Brdu (BD Bioscience 347580, 1:400), NeuN (Millipore, MAB377, 1:800), Arl13b (Santa Cruz, sc-102318, 1:500), Gli2 and Gli3 (gift from S. Scales, Genentech, 1:200).

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) equipped with high-ef ciency fluorochrome specific filter sets for DAPI, Cy2, Cy3 and Cy5. For quantification of the number of Ki-67–expressing, BrdU-stained or Zic1-expressing cells, areas to be counted were traced with a 40× objective lens, and sample frames (40 μm × 40 μm) were selected from at least 10 random but nonoverlapping 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<sup>17</sup>. The probes used were murine Ptch1, Gli1, Gli2 and Cend1. Detailed protocols are available upon request.

Cerebellar GNP culture and proliferation assays. Cerebella from P6 or P7 mice were dissected with Trypsin/DNase (1 mg ml<sup>−1</sup>; Worthington), triturated to obtain a single-cell suspension, and then centrifuged through a 35–65% Percoll gradient (Sigma) according to Yang et al.<sup>13</sup> 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<sup>−1</sup> N-acetyl-d-cysteine and penicillin/streptomycin). We pre-plated GNP s onto poly-d-lysine (100 μg ml<sup>−1</sup>)-coated plates for 1 h at 37 °C twice, and then transferred them to Poly-d-lysine–coated plates for culture. We treated GNP s from control and Gt3 mutants with SAG (Enzo, Lx-270-426) 200 nM, forskolin (Sigma, F9397) 10 μM, a selective Gs antagonist NF449 (Tocris, 1391) 200 μM, GDC-0449 (Selleckchem, S1082) 1 μM, or rolipram 50 μM (R&D, 9095), or we transfected the cells with pDNA3 or pGsaCa for 48 h. For in vitro proliferation assays, we labeled GNP s with BrdU (50 μg ml<sup>−1</sup>) for 48 h 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 s 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 dithiothreitol, 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 r.p.m. 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 from S. Scales, 1:200), α-GTP (NewEast Bioscience, 26904) followed by Protein A/G agarose beads (NewEast Bioscience, 30301) for 1 h. Activated Gt3 and Gt3 proteins were detected by western blotting with Gt3 antibody.

Enzyme-linked immunoassay for cAMP. We plated GNP s on 96-well tissue culture plates and cultured them for 1 d. The cAMP level in the GNP s was assayed following the manufacturer’s protocol (Cell Signaling, 4539). In brief, GNP s were treated with SAG (200 nM), NF449 (100 μM) or forskolin (10 μM) for 24 h. The amount of cAMP in lysates from 8,000 GNP s was measured by ELISA.

Assay for activated Gt3 (Gt3-GTP). We performed activated Gt3-GTP pull-down assays following the manufacturer’s protocol (NewEast Bioscience, 80801). Briefly, GNP s 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 1 μg antibody against the active form Gt3-GTP (NewEast Bioscience, 26906) followed by protein A/G agarose beads (NewEast Bioscience, 30301) for 1 h. Activated Gt3 and Gt3 proteins were detected by western blotting with Gt3 antibody.
RNA-seq data analysis. We isolated RNAs from the cerebella of adult wild-type mice and tumor tissue from individual GFAP-Gnas or Olig1-Gnas mutants and subject it to RNA deep sequencing. RNA-seq libraries were prepared using the Illumina RNA-seq Preparation Kit (Illumina) and sequenced in the HiSeq 2000 sequencer. RNA-seq reads were mapped using TopHat with default settings. TopHat output data was then analyzed by Cufflinks to calculate FPKM values for known transcripts in mouse genome reference and test for changes of gene expression of control and tumors tissues.

Copy number variation analysis. We isolated genomic DNAs from the cerebellum of adult wild-type 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 hybridized them on aCGH arrays (Nimblegen, Mouse CGH 3x20K 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 (≥ 2.03) and loss (≤ −0.5) and significance threshold = 1.0 × 10−5. 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 previously57,58. Unsupervised HCL of MB expression data identified the following four unique sample clusters: WNT-group, SHH-group, group-3 and group-4. Briefly, gene expression data from MB samples were generated by using Affymetrix HTHG-U133A chips (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 analyzed on a nanoString nCounter using a custom 25-gene probe set as previously described29. 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)29.

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)39 in the R environment (v 2.15). Mouse transcripts were mapped to human transcripts using gene orthology predicted by EnsemblCompara GeneTrees60 available on Ensembl BioMart (GRCh38 data set). Subsequently, the expression profiles were analyzed to assign molecular MB subgroup to the mouse tumors, using the AGDEX R package (v 1.0.1)41 and human MB expression data from Northcott et al.37. 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 was also performed using a class prediction algorithm, Prediction Analysis for Microarrays (PAM)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 eigen vectors were used to project the expression profiles of the classified samples onto the vector space spanned by the first two eigen vectors 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 described39. 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-dimensional models and analyzed with Imaris Software (Bitplane) to calculate the volume of tumor tissues.

Intracranial transplantation. Tumor cells isolated from Gt, mutants were plated in GNP culture medium and harvested with 0.25% trypsin and 0.02% EDTA for 2 min, washed twice with Hank’s balanced salt solution (HBSS), and resuspended in Ca2+− and Mg2+-free HBSS. Cell viability was determined by trypsin blue exclusion. Only single-cell suspensions with more than 90% viability were used for in vivo allograft studies. Cells (5 × 103) 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 analyses. All analyses were done using Microsoft Excel or Prism GraphPad 6.00 for Mac OS (San Diego, CA, http://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, we used 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.

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