Disruption of the ciliary GTPase Arl13b suppresses Sonic hedgehog overactivation and inhibits medulloblastoma formation

Sarah N. Bay*,b,1, Alyssa B. Long*, and Tamara Caspary*a,2

*Department of Human Genetics, Emory University, Atlanta, GA 30322; and bGenetics and Molecular Biology Program, Emory University, Atlanta, GA 30322

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Medulloblastoma (MB) is the most common malignant pediatric brain tumor, and overactivation of the Sonic Hedgehog (Shh) signaling pathway, which requires the primary cilium, causes 30% of MBs. Current treatments have known negative side effects or resistance mechanisms, so new treatments are necessary. Shh signaling mechanisms, like those that remove Patched1 (Ptch1) or activate Smoothened (Smo), cause tumors dependent on the presence of cilia. Genetic ablation of cilia prevents these tumors by removing Gli activator, but cilia are a poor therapeutic target since they support many biological processes. A more appropriate strategy would be to identify a protein that functionally disentangles Gli activation and ciliogenesis. Our mechanistic understanding of the ciliary GTPase Arl13b predicts that it could be such a target. Arl13b mutants retain short cilia, and loss of Arl13b results in ligand-independent, constitutive, low-level pathway activation but prevents maximal signaling without disrupting Gli repressor. Here, we show that deletion of Arl13b reduced Shh signaling levels in the presence of oncogenic SmoA1, suggesting Arl13b acts downstream of known tumor resistance mechanisms. Knockdown of ARL13B in human MB cell lines and in primary mouse MB cell culture decreased proliferation. Importantly, loss of Arl13b in a Ptch1-deleted mouse model of MB inhibited tumor formation. Postnatal depletion of Arl13b does not lead to any overt phenotypes in the epidermis, liver, or cerebellum. Thus, our in vivo and in vitro studies demonstrate that disruption of Arl13b inhibits cilia-dependent oncogenic Shh overactivation.

Arl13b | cilia | Shh signaling | medulloblastoma

As the obligate transducer of the pathway, Smo’s activity is key (3). SMOA1 (also known as SmoM2) is a point mutation identified in a BCC patient that results in a Trp → Leu conversion, causing constitutive activation of Smo; it is often used in research to model oncogenic Smo (9). In contrast, bioavailable derivatives of the Smo antagonist cyclopamine are FDA-approved to treat Shh-derived BCCs, but some tumors develop conformational resistance to these drugs through secondary Smo mutations—indicating that molecular treatment is a viable strategy yet to be fully realized (10). Pharmacological manipulation of Smo allows for testing hypotheses related to Smo’s localization and its activation state. The antagonist SANT-1 prevents ciliary accumulation and activation of Smo, whereas cyclopamine traps Smo in the cilium while preventing its activation and downstream pathway activation (Fig. S1) (11, 12). The agonist SAG drives downstream pathway response by directly activating Smo in contrast to Shh ligand, which activates Smo through removal of inhibitory Ptch1.

During normal cerebellar development, Shh acts as a mitogen, stimulating proliferation of cerebellar granule neuron precursors (CGNPs) (13). CGNPs are specified beginning at embryonic day 13.5 (E13.5) in mouse and express the transcription factor Math1 (14, 15). After specification, CGNPs undergo intense Shh-driven, cilia-dependent proliferation before becoming postmitotic and migrating inward to form the internal granular layer of the mature cerebellum (16, 17). Mutations resulting in overactivation and/or deregulation of Shh signaling in these cells, including loss of Ptch1 and the point mutation SMOA1, can lead to MB formation (18).

Significance

Medulloblastoma is the most common malignant pediatric brain tumor. Current therapies are associated with negative side effects, and one-fourth of patients are treatment-resistant or develop tumor progression. Since 30% of medulloblastomas exhibit activation of the Sonic hedgehog (Shh) pathway, much research centers on identifying molecular targets that are able to reduce the high levels of Shh pathway activity that cause tumors. As cilia are required for Shh signaling, we provide evidence that inactivation of a ciliary protein called Arl13b reduces Shh-dependent transcription and proliferation, inhibiting tumor formation in a mouse model of medulloblastoma. Arl13b disruption moderately affects cilia, indicating that Arl13b is a potential candidate for therapeutic drug development.

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1Present address: Genetics Society of America, Bethesda, MD 20814.
2To whom correspondence should be addressed. Email: tcaspar@emory.edu.

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Cilia play a complex role in Shh-mediated carcinogenesis. Mutations that mimic constitutively activated Gli, such as the truncated form of human GLI2 called GLI2ΔN, cause cilial-independent tumors; here, the presence of a cilium protects against tumor formation (19, 20). However, mutations like loss of Ptc1 or activation of Smo cause cilial-dependent tumors as the cilium must transduce the activated signal to produce high GliA/GliR ratios (19, 20). Without the cilium, stable, full-length Gli is produced but is neither activated nor cleaved, so such tumors are suppressed. Loss of cilia in extant tumors from Ptc1<sup>+/−</sup> mice leads to growth arrest and tumor regression (21).

The cilium’s utility as a therapeutic target remains limited. The postnatal requirement of cilia is well established in multiple organ systems. In mouse models, postnatal genetic ablation of cilia results in phenotypes including respiratory difficulties, mistimed growth plate proliferation, ovarian malfunction/infertility, and neurologic/memory problems (22–25). A more effective strategy would be identifying cilia proteins whose loss could lower oncogenic pathway output without complete loss of cilia or signaling (21).

We identified the ciliary GTPase ADP ribosylation factor-like 13b (Arl13b) and characterized its function through studies of null and conditional mouse alleles (26–28). Loss of Arl13b results in ultrastructural defects in the cilium and affects cilial trafficking without resulting in the absence of cilia or Shh signaling (26, 27). Loss of Arl13b results in low-level, ligand-independent, constitutive activation of Shh signaling in the developing neural tube (26) and controls the ligand-gated enrichment of Smo in cilia (27). Analysis of cell fate specification in the neural tube of Arl13b<sup>−/−</sup> and Arl13b<sup>−/+</sup> double mutants showed that Arl13b regulates GliA without affecting GliR (26). Thus, in contrast to mutants that ablate cilia and lose both GliA and GliR production, loss of Arl13b functionally disentangles the relationship among GliA, GliR, and cilia. This mechanistic understanding of Arl13b in Shh signaling led us to study it in an activated-Shh tumor capacity.

Here, we demonstrate that oncogenic Shh overactivation is inhibited by Arl13b disruption in vitro and in vivo. We show that Arl13b functions downstream of activated Smo and demonstrate that knockdown of ARL13B in human MB tumor cells reduces SHH-signaling levels and proliferation. We find that knockdown of ARL13B in primary mouse tumor culture also reduces proliferation and that deletion of ARL13B inhibits MB formation in an established Ptc1-deleted MB mouse model. We demonstrate postnatal deletion of Arl13b does not lead to any overt phenotypes in the epidermis, liver, or cerebellum. Taken together, our data indicate that oncogenic Shh signaling can be reduced by disrupting the cilia gene Arl13b.

**Results**

**The Loss of Arl13b Reduces Shh Pathway Output in the Presence of SmoA1.** We showed that Arl13b regulates Shh signaling at the level of Smo, as well as at a step downstream of Smo (26, 27). To determine Arl13b’s relationship to activated Smo, we generated immortalized Arl13b<sup>−/−</sup> mouse embryonic fibroblasts (MEFs) stably expressing a GFP-tagged, constitutively active form of Smo (Smo<sup>A1-GFP</sup>); by infecting with an adenovirus carrying Cre recombinase, we could induce genetic deletion of Arl13b<sup>−/−</sup> in primary mouse tumor culture also reduces proliferation and that deletion of Arl13b inhibits MB formation in an established Ptc1-deleted MB mouse model. We demonstrate postnatal deletion of Arl13b does not lead to any overt phenotypes in the epidermis, liver, or cerebellum. Taken together, our data indicate that oncogenic Shh signaling can be reduced by disrupting the cilia gene Arl13b.

(A) qRT-PCR of Shh targets Gli1 and Ptc1 shows pathway activation is lowered when Arl13b is deleted in the presence of SmoA1. All data are mean ± SEM of three biological replicates; **P < 0.005 and ***P < 0.0005 between genotypes within a given treatment; **P < 0.005 and ***P < 0.0005 compared with untreated within a given genotype. (B) Western analysis of Gli processing in untreated and Shh-stimulated SmoA1-GFP MEFS with and without Arl13b. (C) Bar graphs show quantification of Gli1 protein in GliA/GliR ratios (19, 20). Without the cilium, stable, full-length Gli is produced but is neither activated nor cleaved, so such tumors are suppressed. Loss of cilia in extant tumors from Ptc1<sup>+/−</sup> mice leads to growth arrest and tumor regression (21).

We further investigated the role of Arl13b downstream of activated Smo by asking whether Gli processing is normal when SmoA1 is present and Arl13b is deleted. We observed decreased Gli1 protein after Arl13b deletion, consistent with the qPCR data (Fig. 1 B and C). We monitored full-length Gli2 protein by Western blot. Deletion of Arl13b lead to a slight downward trend in Gli2FL levels and no change in Gli3FL levels (Fig. 1 B and C). Gli3 is cleaved into its 83 kDa repressor form when Shh ligand is absent (29), and far less repressor is made when the pathway is stimulated. We observed the shift from high to low repressor levels in response to stimulation with Shh CM in SmoA1-GFP cells as well as in SmoA1-GFP Arl13b<sup>−/−</sup> cells, indicating that GliR production is unaffected by the loss of Arl13b regardless of activated Smo (Fig. 1 B and C). Together, these data show full-length Gli proteins either stay steady (Gli2, Gli3) or decrease (Gli1) upon Arl13b deletion while Gli3 cleavage into Gli3R upon pathway stimulation is preserved.

To control for the effect of Smo overexpression in our stable line, we made an analogous cell line in Arl13b<sup>−/−</sup> MEFs that stably expresses SmoWT-GFP at an equivalent level and repeated
all analyses in that line (Figs. S2 B and C, S3 B, and S4). Untreated SmoWT-GFP cells showed no active signaling, indicating that stable overexpression of tagged Smo did not alter normal Smo function. Consistent with our analysis of the SmoaI-GFP ΔARL13b MEFs, we found (i) lower Gli1 and Pch1 transcription, (ii) lower Gli1 levels, (iii) normal Gli2FL and Gli3FL levels, (iv) normal Gli3R processing, and (v) stereotopic Smo localization patterns in SmoWT-GFP ΔARL13b MEFS, confirming that deletion of ARL13b lowers the Shh transcriptional response to Shh CM/SAG regardless of Smo activation.

**ARL13B Knockdown Reduces SHH Signaling and Proliferation in Human MB Cell Lines.** To test whether manipulating ARL13B has similar effects in human cells as in mouse cells, we knocked down ARL13B in two human MB cell lines: DAOY and D556 (30, 31). The DAOY cell line was isolated from a desmoplastic MB—a histological variant of MB associated with activation of SHH signaling. D556 cells are MYCC-amplified, derived from an anaplastic MB, and often used in culture experiments as a “non-SHH” tumor cell line. After confirming that DAOY cells were Shh-sensitive while D556 were not (Fig. S5A), we hypothesized that disruption of ARL13B would affect DAOY but not D556 cells. We infected cells with a lentiviral shRNA targeting ARL13B or a scrambled control and assayed SHH target gene transcription in response to ligand by qRT-PCR (Fig. 2 A and B and Fig. S5B). DAOY cells up-regulate GLI1 and PTCH1 in response to Shh CM, while D556 cells do not. Knockdown of ARL13B reduces the stimulated SHH response in DAOY cells and, surprisingly, in D556 cells regardless of pathway stimulation.

To test whether the knockdown of ARL13B affected proliferation in these cells, we examined BrdU incorporation. ARL13B knockdown resulted in a significant decrease in proliferation in both DAOY and D556 cells (Fig. 2 C and D). Together, these data show that loss of ARL13B causes down-regulation of SHH signaling and reduces proliferation in human MB cell lines.

**Arl13b Knockdown Reduces Proliferation of Mouse MB Cells.** We next turned to an ex vivo system to investigate whether Arl13b functions in tumor cell maintenance by knocking down Arl13b via lentiviral shRNA in primary mouse MB cells and assaying for proliferation. We used a well-established MB model expressing Smoa1 under the control of the neurod2 promoter, known as nD2::SmoA1 (32). We infected cells with lentiviruses carrying one of two shRNAs against Arl13b (designated 442 or 968) or a scrambled control and compared tumors left unstimulated or treated with ShhN. We monitored proliferation via BrdU incorporation. Modest knockdown of Arl13b in these cells reduced proliferation with or without ShhN stimulation (Fig. 3 A and B and Fig. S6).

Since constitutive activation of Smo is only one tumorogenic mechanism, we repeated this experiment in a Pch1-deleted mouse model of MB (33, 34). We used Math1-CreER to induce Pch1 postnatal recombination and deletion specifically in CGNs through tamoxifen treatment at E14.5 (referred to as Pch1^ΔMath1-Cre-E14.5; animals). We labeled cells in S phase through BrdU incorporation and discovered few stained cells, indicating the proliferation rate was too low for us to measure differences between genotypes and conditions. To circumvent this, we monitored cells in any active cell cycle phase using Ki67. Knockdown of Arl13b resulted in significantly less Ki67 staining than the scrambled control regardless of treatment with ShhN (Fig. 3 C and D). Taken together, these data show that loss of Arl13b can reduce proliferation in primary culture of cilia-dependent tumors.

**Loss of Arl13b in the Developing Cerebellum Inhibits MB Formation.** Pch1^ΔMath1-Cre-E14.5 animals developed tumors quickly and robustly, so we used this model to test whether the loss of Arl13b could prevent MB formation in vivo. We compared a control tumor model in which we deleted Pch1 to an experimental model in which we concurrently deleted both Pch1 and Arl13b. We used Math1-CreER to induce deletion via tamoxifen treatment at E14.5. We followed control Pch1^ΔMath1-Cre-E14.5 mice (n = 23) and experimental Arl13b^−ΔMath1-Cre-E14.5 mice (n = 23), monitoring them for head doming, ataxia, and weight loss as symptoms of MB formation. Only 9% (2/23) of Pch1-deleted mice survived to the study endpoint of 150 d, with a median survival of 99 d (Fig. 4 A and B). In contrast, 78% (18/23) of Pch1 Arl13b-deleted animals survived to the endpoint with no animal dying before 124 d (Fig. 4 A and E). Twenty-two percent of experimental animals developed tumors (Fig. 4 F), but these tumors formed later than in the control mice. In contrast to control animals, these tumors did not wholly disrupt cerebellar structure, leaving some intact internal granule layer (IGL) visible (Fig. 4 B and F).

The fact that the tumors in the experimental animals did not affect the entire cerebellum raised the possibility that they derived from a subpopulation of Pch1-deleted cells that did not also delete Arl13b. To investigate, we looked for the presence of Arl13b-positive cilia within experimental tumors. We found that, like control tumors, the late-forming tumors in the experimental animals were Arl13b-positive (Fig. 4 C and G), indicating that Cre-mediated recombination had not occurred in those cells or that Arl13b protein had not turned over. In contrast, the remaining IGL of tumor-positive experimental animals displayed few Arl13b-positive cilia—similar to the IGL of surviving experimental animals (Fig. 4 E, H, and I). Previous reports documented...
that distinct floxed alleles recombine discordantly (35, 36), so we interpret these tumors as deriving from a subpopulation of cells in which Ptch1 deleted more efficiently than Arl13b. These data indicate that the loss of Arl13b disrupts Ptch1-deleted MB formation and is likely to function cell autonomously.

Postnatal Function of Arl13b. Embryonic loss of Arl13b is lethal, and tissue-specific embryonic deletion of Arl13b in the developing kidney leads to cystic kidneys and death (26, 37, 38). To test whether Arl13b continues to be required postnatally, we induced Arl13b deletion by administering tamoxifen to Arl13b^{flox/flox} and Arl13b^{flox/flox}; CAGG-CreER mice with tamoxifen at P14, P16, and P18 and followed them until P60. In contrast to the earlier time point, these animals (n = 11), noted as Arl13b^{flox/flox};CAGG-CreER^{−/−}, had similar body sizes to Cre-negative littermates (n = 7) (Fig. S7A). Cre-positive animals displayed no obvious ataxia or motor control issues, and females were capable of reproduction. At P60, we performed a gross necropsy and found no visible defects in the heart, lungs, reproductive organs, or brain. We further examined kidney and cerebellar morphology via H&E staining and found size and foliation were comparable between the two genotypes (Fig. 5 B and C).

Previous work defined a critical window for kidney development ending around P14 (39, 40), so we next treated Arl13b^{flox/flox} and Arl13b^{flox/flox}; CAGG-CreER mice with tamoxifen at P14, P16, and P18 and followed them until P60. In contrast to the earlier time point, these animals (n = 11), noted as Arl13b^{flox/flox};CAGG-CreER^{−/−}, had similar body sizes to Cre-negative littermates (n = 7) (Fig. S7A). Cre-positive animals displayed no obvious ataxia or motor control issues, and females were capable of reproduction. At P60, we performed a gross necropsy and found no visible defects in the heart, lungs, reproductive organs, or brain. We further examined kidney and cerebellar morphology via H&E staining (Fig. 5 C, C’, D, and D’). The CAGG-CreER allele is expressed ubiquitously, and we observed substantial yet variable deletion upon tamoxifen induction. We found low-level Arl13b protein in the cerebellum, as expected, but residual Arl13b protein expression in the kidneys of Arl13b^{flox/flox};CAGG-CreER^{−/−} mice (Fig. S7 B and C). We observed widely variable Arl13b loss in kidney across the Arl13b^{flox/flox};CAGG-CreER^{−/−} cohort—indicating either inefficient Cre–lox recombination or protein turnover in the kidney. We observed a few small cysts largely localized to the renal cortex in the Arl13b^{flox/flox};CAGG-CreER^{−/−} animals (Fig. 5C’); this agrees with observations that postnatal loss of cilia genes can result in slow-growing renal cysts and reinforces the importance of Arl13b’s role in ciliogenesis in the kidney (38, 40). We found recombination of the floxed allele in ear punch and liver DNA, indicating that, like cerebellum, Arl13b is depleted in these tissues (Fig. S7D). Depleted animals had normal cerebellar morphology, as in the Arl13b^{flox/flox};CAGG-CreER^{−/−} animals (Fig. 5 D and D’). Taken together, these data argue that Arl13b depletion after P14 in mouse does not lead to any overt phenotypes in liver, skin, or cerebellar tissue.

Discussion

Our data show that deletion of Arl13b reduced Shh signaling activity and inhibited tumor formation in vivo. Arl13b deletion diminished the Shh transcriptional response when the pathway was active. Control: Ptch1^{Math1-Cre-El14.5}. Experimental: Arl13b^{Math1-Cre-El14.5}. (A) Graph shows significant difference in survival curves between control and experimental mice. ***P < 0.0001; log-rank test. (B) H&E staining of a representative control brain with MB. (C) Image at 100x of tumor from B shows Arl13b-positive cilia in white. (D) H&E staining of a surviving experimental brain with no tumor. (E) Image at 100x of IGL from D shows few Arl13b-positive cilia in white. (F) H&E staining of a tumor-positive experimental brain shows normal cerebellar structure in addition to tumor. G+ show the difference in Arl13b staining (white) between tumor tissue (G at 100x) and normal IGL (I at 100x). H shows the boundary between tumor and IGL at 60x. Solid line: identifiable cerebellar foliation; dotted line: tumor. (Scale bars, 1 cm.)
stimulated with Shh ligand, drug agonist (SAG), or an activating Smo mutation. We observed the same trend in MEFs, human MB cell lines, and primary mouse MB cells, indicating that the effect is robust in distinct cell types and is applicable in both mouse and human cells. Furthermore, we found that processing of the Gli transcription factors in the absence of Arl13b is distinct from their established processing in the absence of cilia (41–43). Taken together, our data provide proof of principle for Arl13b inhibition as a potential therapeutic option for Shh-derived cancers, thus warranting further studies on the ability of Arl13b disruption to reverse extant tumors in vivo. More broadly, our data suggest that a strategy of targeting ciliary proteins that reduce Shh response with minimal impact on cilia is effective in antagonizing Shh-activated tumors. Our work advances previous findings that the reduction of GliA through the disruption of cilia could work, in principle, to prevent MB (19, 21).

The loss of Arl13b reduces oncogenic signaling output through a distinct mechanism from the loss of cilia. When cilia are lost, neither GliA nor GliR are produced, resulting in Westerns detecting increased GliF (41–43). Increased GliF in the absence of cilia is interpreted as Gli protein that cannot be activated. In contrast, we find loss of Arl13b leaves Gli2F and Gli3F levels unchanged upon stimulation, which is reflected by the lowered Shh transcriptional response; furthermore, loss of Arl13b preserves the normal processing of Gli3R regardless of which is activated. In our previous work demonstrating that Arl13b regulates Shh signaling through at least two steps: (i) in the ligand-dependent enrichment of ciliary Smo and (ii) downstream of Smo (26, 27). Our analysis of Arl13b deletion in SmoA1-GFP MEFS and in primary SmoA1 MB tumor culture specifies that Arl13b functions downstream of activated Smo and is consistent with Arl13b being required for robust pathway activation. Future work may identify distinct Arl13b effectors more specific in inactivating specific regulatory steps of Shh signal transduction—or those that would uncouple Arl13b’s role in regulating Shh signaling from its role in cilia architecture (46, 47). Indeed, one proposed effector of Arl13b is the phosphatase Inpp5e, which was recently shown to contribute to tumor maintenance (48, 49).

Since Arl13b functions both at a step involved in Smo ciliary enrichment and at a step downstream of activated Smo—and could have other undiscovered regulatory roles—tumors might need to develop multiple resistance mechanisms to overcome Arl13b inhibition. Arl13b-targeted therapies could be appropriate to use in combination with Smo inhibitors and could help combat the canonical secondary Smo mutations that promote tumor resistance to Smo-inhibiting drugs. In fact, recent work with Arl13b in gastric cancer proposed that Arl13b directly interacts with Smo for stabilization and trafficking and that loss of Arl13b results in Smo degradation (50). This points directly at a potential mechanism for how Arl13b controls Smo ciliary enrichment, but whether this mechanism is related to the role we establish for Arl13b downstream of activated Smo, or if it is specific to gastric cancer, remains unknown. In MEFS, we observed ciliary SmoWT-GFP and SmoA1-GFP in the absence of Arl13b.

Most SHH-activated tumors arise from mutations in PTCH1 or SMO, but some arise from mutation of SUFU, which is required for GLI processing, and GLI1/2 amplification (51–53). Mutations that do not rely on trafficking through the cilium to stimulate the pathway, such as those that mimic activated Gli (like GLI2ΔN), give us insight into the mechanism of tumorigenic signaling (19, 20). In these cases, when the mutations are effectively “downstream” of the cilium, removing the cilium increases MB incidence, specifically due to loss of GliR (19). Since deletion of Arl13b preserves GliR, it could plausibly have an effect on such “cilia-independent” mutations in the Shh pathway, though this remains to be tested.

We were surprised to find that ARL13B knockdown had similar effects in D556 cells and D456 cells, as D556 cells are sometimes used to model non-SHH MB. This could indicate that SHH signaling is overactivated in this cell line as well, consistent with the ligand insensitivity we observed, or this could also indicate that ARL13B manipulation may be efficacious in a wider variety of MBs than originally hypothesized.

We used a ubiquitous Cre line to deplete Arl13b postnatally; we observed efficient deletion in the cerebellum, epidermis, and liver but variable loss of Arl13b protein in the kidney. Despite the critical role of Arl13b during development, we found most organ systems were grossly normal, with the exception of the kidney. Our data add to previous findings that Arl13b functions differently in the kidney than other tissues. Kidneys are the only tissue in which Arl13b deletion results in a lack of cilia (38, 54), and they are the tissue in which we did not induce a robust lowering of Arl13b expression using Cre–lox. While more thorough work on the postnatal roles of Arl13b is needed, these initial findings suggest that Arl13b inhibition may be an appropriate strategy against MB provided the kidney issues can be circumvented. This may be possible if effectors specific to Arl13b’s role are identified or if Arl13b’s role relative to kidney homeostasis over time is better understood.

Our work proposes depletion of Arl13b as a strategy to lower oncogenic Shh signaling by decoupling the regulation of GliA and GliR. It will be important to determine whether there are other such proteins whose inhibition would exploit this strategy. Future experiments addressing the efficacy of Arl13b disruption in existing tumors in vivo remain to be done. Recent research showed that the stromal environment surrounding a tumor plays an important role in tumor development and maintenance (55), underscoring the importance of in vivo experiments to follow our
work. Our data that targeting Arl13b reduces tumorigenic Shh signaling lays the foundation for future work into drug development and investigating the role of Arl13b in other Shh-derived tumors, BCC and MB share common tumorigenic mechanisms, so it is likely that Arl13b inhibition would similarly impact BCC and should be investigated.

Materials and Methods

Animal work was carried out under Institutional Animal Care and Use Committee-approved protocols at Emory University, and all cell culture work was done under an Emory Environmental Health and Safety-approved biosafety protocol. Lentivirus was produced using the Sigma MISSION Lentiviral Packaging Mix (SHP001) and Promega FuGENE 6 (E2691) according to the manufacturer’s instructions. Coverslips were mounted in ProLong Gold antifade reagent (P36934; Thermofisher Scientific) and imaged using an Olympus Fluoview FV1000 confocal microscope and Olympus Fluoview fv4.2 or a Leica CTR6000 microscope with SimplePCI. All statistical analysis was done using GraphPad Prism 7 software.

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