Resistance to Targeted Therapy in Sonic Hedgehog Subgroup Medulloblastoma: Mechanisms and Treatment Strategies

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Resistance to Targeted Therapy in Sonic Hedgehog Subgroup Medulloblastoma:
Mechanisms and Treatment Strategies

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by
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Resistance to Targeted Therapy in Sonic Hedgehog Subgroup Medulloblastoma: 
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ABSTRACT

Aberrant activation of the Sonic Hedgehog (Shh) signaling network is implicated in many human cancers, including the most common cancer, basal cell carcinoma (BCC) and the brain tumor medulloblastoma. Suppressing Shh signaling is thus a promising strategy in oncology. The largest and most clinically advanced group of Shh signaling inhibitors comprises selective antagonists of the pathway component Smoothened (Smo). In 2012, the Food and Drug Administration approved the first Smo antagonist, and others are now approved or in clinical trials. However, there is already evidence that some patients can have primary or develop secondary resistance to therapy. Accordingly, a more comprehensive understanding of resistance mechanisms and alternative treatment approaches are needed. Here, we describe a genome-wide transposon mutagenesis screen to identify candidate resistance genes for Smo antagonists using an in vitro model of Shh-dependent medulloblastoma. Top hits from the screen include Suppressor of fused (Sufu), a known resistance gene and negative regulator in Shh signaling, and Oral-facial-digital syndrome 1 (Ofd1), a gene associated with an X-linked developmental syndrome. Independent gain- and loss-of-function experiments confirm Ofd1 as a bona fide resistance gene. Ofd1 mutant cells have
reduced numbers of primary cilia, which are necessary for transducing canonical Shh signaling. Reduction of Kif3a and Ift88, two other cilia genes, also causes resistance. Strikingly, resistant cilia mutants are still dependent on active Shh signaling downstream of Smo. These mutants lack the truncated repressor form of the Shh transcription factor Gli2, but maintain full-length Gli2 levels and therefore shift the overall balance of transcriptional activators and repressors toward pathway reactivation. Importantly, we present evidence that resistance by loss of primary cilia may have clinical relevance. Subcutaneous medulloblastoma tumors in mice that acquire de novo resistance to Smo inhibition exhibit decreased numbers of primary cilia compared to tumors that remain sensitive. Additionally, resistant BCCs from patients treated with Smo antagonists have significantly more cilia gene mutations compared to untreated BCCs.

Recognizing the need for more options to treat resistant tumors, we carried out a high-throughput small molecule screen in Shh-dependent medulloblastoma cells. From a set of over 900 small molecules, we identify histone deacetylase (HDAC) inhibitors as a class of promising therapeutics. While not all HDAC inhibitors are effective, we present some with similar chemical structures that work consistently within the nanomolar range across cell lines that are both sensitive and resistant to Smo inhibitors. Preliminary results indicate that inhibition of HDACs works within the Shh signaling axis and that specific HDACs may play a role in resistant human tumors. Together, these studies reveal new resistance mechanisms and explore the development of next-generation treatments in Shh-dependent tumors.
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CHAPTER 1: INTRODUCTION

Contributions:
Ekaterina Pak wrote this chapter, portions of which are excerpted from: Pak, E. and Segal, R.A. Hh signal transduction: key players, oncogenic drivers, and pharmacologic targets. Developmental Cell (invited manuscript under review).
The *hedgehog* (*hh*) gene was first reported in 1980 and was named for its role in the embryonic patterning of *Drosophila melanogaster* (*Drosophila*), since mutants displayed a pattern of larval denticles that resembled the bristles of a hedgehog (Nusslein-Volhard & Wieschaus, 1980). Subsequently, three mammalian homologs of the Hh family of secreted proteins were identified: Indian Hedgehog (Ihh), Desert Hedgehog (Dhh), and Sonic Hedgehog (Shh) (reviewed in Ingham & McMahon, 2001). Although the processing and signaling of mammalian Hh proteins is similar, their expression patterns and developmental contributions differ. Among other roles, Ihh is recognized for functions in bone development, while Dhh is involved in peripheral nerve and reproductive organ development. By comparison, the roles of Shh are relatively diverse, encompassing regulation of the developing nervous system, limbs, muscles, and organs like the lungs and kidneys. Shh achieves its multitude of functions by acting as a mitogen and/or a morphogen. Aberrant Shh signaling can lead to a variety of birth defects or development of cancer (reviewed in Cohen, 2010 and in Barakat et al., 2010).

Here, we review the current mechanistic picture of mammalian Hh pathway regulation, from ligand production to transcriptional output. We also discuss Hh signaling in cancer, with a focus on basal cell carcinoma (BCC) and medulloblastoma (MB). Finally, we provide an update on the development and use of targeted therapies for oncogenic Hh signaling. For the remainder of this thesis, we refer to Hh signaling for general concepts on the pathway and Shh signaling where it is more relevant to specify this ligand.
**Hh ligand production**

Hh ligand is synthesized as a 45-kDa precursor protein that is auto-proteolytically cleaved into a ~19-kDa amino terminal fragment and a ~25-kDa carboxy terminal fragment. The amino terminal fragment is modified by a palmitate at its N-terminus and cholesterol at its C-terminus (reviewed in Farzan et al., 2008). The resulting dually-lipidated ligand (referred to as “Hh” or “Shh” from here on) forms multimers and is released by the transmembrane protein Dispatched into the extracellular space.

The lipid modifications on Hh are important for its multimerization, movement in extracellular space, gradient distribution, and activity at the receiving cell. Adding to this established paradigm of Hh ligand biogenesis, a recent study proposed that Shh ligands are not exclusively modified by palmitic acid at their N-termini, but by a variety of different fatty acids that affect intracellular trafficking in the producing cell and ligand potency at the receiving cell (Long et al., 2015). The types of modifications depend on the growth and metabolic context of the producing cells. Changes in the cellular environment such as during early development may directly translate into modified ligands for executing specific long- or short-range functions.

**Hh ligand reception**

Secreted Hh proteins can move many cell diameters from the releasing cell, establishing a concentration gradient of ligands and often controlling developmental outcomes in a concentration-dependent manner. The concentration gradient established by secreted Hh ligands must be paralleled by a reception system capable of converting the graded signal into appropriate levels of pathway output.
The primary receptor for Hh ligand is the twelve-pass transmembrane protein, Patched 1 (Ptch1). Ptch1 is unusual compared to other signaling pathway receptors because it does not act as a signal transducer to activate the pathway upon ligand binding, but rather blocks pathway activity in the absence of ligand. When Hh ligand binds Ptch1, both ligand and receptor are internalized and degraded. Thus, ligand binding not only removes pathway repression by Ptch1, but also limits the half-life of ligand. The mechanisms by which Ptch1 is removed from the cell surface upon ligand binding are not fully understood. Studies in mice demonstrate that Ptch1−/− embryos die by embryonic day 10.5 (E10.5), while heterozygous Ptch+/− mice develop tumors characteristic of over-active Hh signaling (Goodrich et al., 1997).

Besides Ptch1, other receptors for Hh ligands modulate pathway activation (reviewed in Beachy et al., 2010). Positive coreceptors include Cdo, Boc, and the vertebrate specific Gas1. Another vertebrate specific coreceptor, Hhip, acts as a negative regulator of Hh signaling. Many positive coreceptors (Cdo, Boc, and Gas1) are transcriptionally repressed, while negative receptors (Ptch1 and Hhip) are activated following pathway activation. Additionally, proteoglycans function as coreceptors with either positive or negative effects on Hh signaling depending on their unique protein and sugar composition. The resulting network of receptors and feedback loops helps cells properly interpret the duration and graded level of Hh signaling (reviewed in Ribes & Briscoe, 2009).
**Hh pathway activation**

In the absence of Hh ligand, Ptch1 inhibits Smoothened (Smo), a seven-pass transmembrane protein that functions as a potent pathway activator. The mechanisms by which Ptch1 inhibits Smo are unknown. A popular model suggests that Ptch1 does not physically interact with Smo, but rather regulates the transport and/or synthesis of a small molecule (or molecules) that affect Smo activity (Taipale et al., 2002). Upon Shh ligand binding and Ptch1 degradation, Smo becomes phosphorylated by casein kinase 1 (CK1) and G-protein coupled receptor kinase 2 (GRK2), causing Smo to assume an activated conformation and move into the primary cilium (Chen et al., 2011a).

The relay of Hh signaling downstream of activated Smo is not yet completely understood. Ultimately, signal transduction results in expression of a Hh transcriptional program mediated by activator and repressor forms of the Gli transcription factors. Signal transducers functioning between Smo activation and Gli transcription include the negative pathway regulator, Suppressor of fused (Sufu), and the kinesin family protein Kif7. Gli interactions with these two proteins and post-translational modifications by other pathway regulators described below establish a highly complex but well regulated network that ultimately controls cell fate (Figure 1.1).
Figure 1.1

**A.** In the absence of Shh ligand, Ptch1 localizes to the primary cilium and inhibits Smo activation and ciliary localization. Low levels of Kif7, Sufu, and full-length Gli (Gli_{FL}) enter the primary cilium, which promotes Gli_{FL} processing into repressor form (Gli_{R}) after phosphorylation by PKA, GSK3, and CK1. The orphan GPCR, Gpr161, localizes to the primary cilium and promotes production of cAMP, likely via G_{s}-mediated activation of adenylyl cyclase. The resulting formation of Gli_{R} blocks transcription of Shh target genes.

**B.** When Shh ligand binds Ptch1, both ligand and receptor are internalized and degraded. Smo is phosphorylated by CK1 and GRK2, assumes an active conformation, and moves into the primary cilium. Kif7, Sufu, and Gli also accumulate in the primary cilium, where activated Smo promotes Sufu-Gli dissociation and activation of Gli (Gli_{A}). Gli_{A} shuttles into the nucleus and induces target gene transcription. Pathway activation also removes Gpr161 from within the primary cilium and suppresses production of Gli_{R}.

**Figure 1.1 Vertebrate Shh signal transduction.** (A) In the absence of Shh ligand, Ptch1 localizes to the primary cilium and inhibits Smo activation and ciliary localization. Low levels of Kif7, Sufu, and full-length Gli (Gli_{FL}) enter the primary cilium, which promotes Gli_{FL} processing into repressor form (Gli_{R}) after phosphorylation by PKA, GSK3, and CK1. The orphan GPCR, Gpr161, localizes to the primary cilium and promotes production of cAMP, likely via G_{s}-mediated activation of adenylyl cyclase. The resulting formation of Gli_{R} blocks transcription of Shh target genes. (B) When Shh ligand binds Ptch1, both ligand and receptor are internalized and degraded. Smo is phosphorylated by CK1 and GRK2, assumes an active conformation, and moves into the primary cilium. Kif7, Sufu, and Gli also accumulate in the primary cilium, where activated Smo promotes Sufu-Gli dissociation and activation of Gli (Gli_{A}). Gli_{A} shuttles into the nucleus and induces target gene transcription. Pathway activation also removes Gpr161 from within the primary cilium and suppresses production of Gli_{R}. 
Gli transcription factors

Graded levels of Hh signaling trigger the expression of different sets of response genes, depending on the ratio of Gli activator (GliA) and Gli repressor (GliR) forms (reviewed in Hui & Angers, 2011). In mammals, there are three Gli gene family members: Gli1, Gli2, and Gli3. Gli1 is a Hh response gene for which the corresponding protein exists only as a transcriptional activator and functions in a positive feedback loop upon pathway activation. Gli2 functions primarily as a transcriptional activator, while Gli3 serves as the primary transcriptional repressor.

There are multiple regulatory inputs that control GliA and GliR functions. In the absence of Hh ligand, full-length Gli (GliFL) is phosphorylated at multiple sites by protein kinase A (PKA), glycogen synthase kinase 3 (GSK3), and CK1. Hyperphosphorylated GliFL is bound by the adaptor protein β-TrCP, and the resulting complex is ubiquitinated by a Cul1-based E3 ligase and targeted for proteosomal processing to form a truncated transcriptional repressor (GliR). Alternatively, GliFL may also be completely degraded by the proteasome, facilitated by Spop-mediated Cul3-based E3 ligase ubiquitination. Gli2FL phosphorylation predominantly induces complete proteasomal degradation, while phosphorylated Gli3FL is more efficiently processed into Gli3R (Pan & Wang, 2007). Smo activation blocks Gli proteolysis and simultaneously promotes GliA function.

Modulators of Gli activity

The negative pathway regulator, Sufu, functions between Smo and the Gli transcription factors. Sufu−/− mouse embryonic fibroblasts display ligand-independent
pathway activation, while Sufu−/− embryos die by E9.5 with phenotypic over-activation of Hh signaling (Svärd et al., 2006). Sufu directly interacts with and sequesters GliFL in the cytoplasm. Sequestration of GliFL prevents its nuclear translocation and promotes phosphorylation and processing of GliFL into GliR (Humke et al., 2010). Sequestration also stabilizes full-length Gli2 and Gli3, protecting them from proteosomal degradation and thus maintaining a pool of available Gli proteins for Hh signal transduction (Wang et al., 2010). Challenging the traditional cytoplasm-centric roles of Sufu, some have suggested that Sufu can regulate Gli activity in the nucleus (Lin et al., 2014).

Like Sufu, Kif7 is an evolutionarily conserved component of Hh signaling that modulates Gli function downstream of Smo. Kif7 interacts with all three Gli proteins, and is associated with both positive and negative regulatory roles in Hh signaling. Kif7 localizes at the base of the primary cilium in the absence of Hh ligand, but moves into the primary cilium and is important for Gli2 and Gli3 accumulation at the cilium tip when the pathway is stimulated (Endoh-Yamagami et al., 2009; Liem et al., 2009). Kif7−/− mice display phenotypes consistent with loss of Gli3R function, thus indicating a role for Kif7 in negative regulation of Hh signaling. Intriguingly, the Anderson group recently proposed that a major role for Kif7 in Hh signaling is to control cilium length and architecture (He et al., 2014a). In this role, Kif7 ensures that a single cilium tip compartment is established where Gli and Sufu can localize for signal transduction. Integrating the functional contributions of Kif7 to cilium assembly with the dynamic movement of Kif7 into the primary cilium upon Hh ligand stimulation will be important to comprehensively understand the multifaceted roles of Kif7 in Hh signaling.
Gli phosphorylation is another mechanism of regulation. Recently, atypical protein kinase C \( \text{I}/\lambda \) (aPKC-\( \text{I}/\lambda \)) was identified as a Gli regulator involved in a positive feedback loop that potentiates Hh activation (Atwood et al., 2013). Phosphorylation of Gli1 by aPKC-\( \text{I}/\lambda \) promotes maximal Gli1 DNA binding and transcriptional activation. In turn, active Hh signaling promotes both the expression and activation of aPKC-\( \text{I}/\lambda \). Gli1 is also phosphorylated by AMP-activated protein kinase (AMPK), which induces Gli1 degradation (Li et al., 2015). Importantly, regulation of Gli1 by the energy sensor AMPK links cellular metabolic state to Hh transcriptional output, which may be crucial for the developmental roles of this pathway.

Another kinase that modulates Gli function is cyclic adenosine monophosphate (cAMP)-dependent PKA. Sufu, Gli2, and Gli3 can be phosphorylated by PKA, which functions as a master negative regulator of the Hh pathway. Sequential phosphorylation of Sufu by PKA and GSK3 stabilizes Sufu in a complex with Gli2/3 that moves into the primary cilium in response to Hh ligand (Chen et al., 2011b).

As described above, GliFL proteolysis is also regulated by PKA. The unique patterns of Gli2/3 phosphorylation may be important for converting differences in Hh signal strength into discrete states of Gli activity. PKA phosphorylation of six conserved serine residues (P1-6) on Gli2/3 drives GliR and inhibits GliA formation (Niewiadomski et al., 2014). Interestingly, selective phosphorylation of the first four PKA sites (P1-4) is sufficient to target processing of GliFL into GliR, while inhibition of GliA requires phosphorylation of all six PKA sites. Smo activation reduces phosphorylation at P1-6, which allows PKA-independent Gli phosphorylation at a different cluster of Ser/Thr sites (Pc-g) and results in full transcriptional activation of Gli2/3.
Many inputs modulate PKA activity and thereby affect Hh pathway output. For example, production of cAMP by adenylyl cyclase and degradation of cAMP by phosphodiesterases can promote and attenuate PKA activity, respectively. In a pivotal study, production of cAMP was mechanistically coupled to Shh signaling via the orphan GPCR, Gpr161 (Mukhopadhyay et al., 2013). In the absence of Shh ligand, Gpr161 localizes to the primary cillum and promotes increased levels of cAMP, probably via Gαs-mediated activation of adenylyl cyclase. In the presence of Shh ligand, Gpr161 is removed from cilia, preventing cAMP production and thus promoting pathway activation. Importantly, Gpr161 unifies many components of Shh signaling: ligand stimulation, PKA regulation, and roles of the primary cillum, which are reviewed in more detail below.

**The primary cillum in Hh signaling**

Primary cilia play a central role in vertebrate Hh signaling. The primary cillum consists of a basal body that is anchored to the plasma membrane and an axoneme, which protrudes into the extracellular space. The basal body is derived from the mother centriole at the end of cell division. The axoneme is composed of nine microtubule pairs and is built from the basal body. The assembly, disassembly, and maintenance of cilia are executed by intraflagellar transport (IFT) proteins and their associated kinesin and dynein motors.

All core components of vertebrate Hh signaling, including Ptch1, Smo, Sufu, Kif7, and Gli proteins, dynamically localize to the primary cillum. Upon Hh ligand binding, Ptch1 exits the base of the primary cillum, while Smo accumulates within the cillum. While ciliary accumulation of Smo correlates with Hh pathway activation, it is not
sufficient for signal transduction and additional mechanisms described above are required. Pathway activation also promotes Sufu-Gli complex movement into the primary cilium where they dissociate, resulting in concurrent enrichment of Gli in the distal tip of the cilium and Gli translocation to the nucleus. Smo translocation and Gli dissociation demonstrate positive regulation of Hh signaling via the primary cilium. Cilia also participate in pathway inhibition by mediating proteolytic processing of GliFL into GliR. Mice with defective or absent cilia display functional loss or altered ratios of GliA/R (Goetz and Anderson, 2010).

Precise localization of proteins within the primary cilium is important for signal transduction. For example, the EvC zone, named after Ellis-van Creveld Syndrome, is located at the base of the primary cilium and defines a distinct compartment where Smo accumulates in response to Hh ligands by binding to the proteins EVC and EVC2. Anchoring of the EVC-EVC2 complex to the EvC zone is required for activation of Gli2 but not for regulating levels of Gli3R (Pusapati et al., 2014). This type of GliA/GliR signaling bifurcation downstream of Smo has also been demonstrated for the ciliary basal body-localized protein Dlg5 (Discs large, homolog 5) (Chong et al., 2015). Upon ligand stimulation, Dlg5 interacts with Smo to promote Kif7 and Gli2 ciliary accumulation and Gli2 activation, but Dlg5 is not required for suppression of GliR formation. Subciliary localization of Hh signaling regulators such as Dlg5 and EvC complex proteins may help coordinate the contributions of GliA/GliR functions in response to different levels of pathway activation.

Recent work has highlighted how the lipid composition of the primary cilium contributes to trafficking and signaling of Hh pathway components. The
phosphoinositide PI(4)P is enriched in the ciliary membrane, whereas both PI(4)P and PI(4,5)P2 are found in the plasma membrane (Chavez et al., 2015; Garcia-Gonzalo et al., 2015). This distinct lipid composition of the primary cilium is maintained by the cilium-localized phosphatase, Inpp5e, which dephosphorylates ciliary PI(4,5)P2 into PI(4)P. Loss of Inpp5e results in PI(4,5)P2 accumulation at the ciliary membrane, which in turn recruits and maintains Tubby-like protein 3 (Tulp3) and its interacting intraflagellar transport (IFT-A) proteins in the primary cilium. Importantly, the negative Shh regulator Gpr161, discussed above, uses Tulp3 and IFT-A complex to traffic into cilia and therefore also accumulates in the primary cilium upon Inpp5e loss. Ciliary accumulation of Gpr161 in Inpp5e mutants is accompanied by increased cAMP levels and hindered Shh target gene activation. Inpp5e loss also impairs Gli3 accumulation at the ciliary tip following pathway stimulation, but does not affect Smo trafficking. Thus, Inpp5e establishes a distinct phosphoinositide composition in the primary cilium, which limits ciliary accumulation of negative Hh regulators and therefore permits ligand-induced signal transduction.

**Hh signaling in basal cell carcinoma**

An early link between Hh signaling and cancer was the discovery that inherited loss-of-function mutations in the *PTCH1* gene are responsible for nevoid basal cell carcinoma syndrome (NBCCS), also called Gorlin syndrome (Barakat et al., 2010). This autosomal dominant disease is characterized by predisposition to BCCs with *PTCH1* loss of heterozygosity in the skin, as well as higher incidence of other neoplasms, especially MB.
Although Gorlin syndrome is rare, sporadic BCC is the most common human cancer. Sporadic BCC is associated with UV exposure and is also driven by aberrant activation of Hh signaling. High frequencies of inactivating mutations in PTCH1 (~70-90%), and to a lesser extent activating mutations in SMO (~10-20%), are reported in sporadic BCC (Atwood et al., 2015; Reifenberger et al., 2005; Sharpe et al., 2015). SUFU mutations are rare, and often accompanied by PTCH1 and/or TP53 mutations. Interestingly, targeted Sufu inactivation in mouse skin results in G2/M cell cycle arrest and little or no formation of BCCs (Li et al., 2014). For all BCC cases, the common factor is constitutive activation of the Hh pathway.

The majority of BCC cases are successfully treated with local topical therapies and surgery. In contrast, rare, locally advanced BCCs that can no longer be controlled by surgery and/or radiation as well as metastatic BCCs are associated with significant morbidity and mortality.

Hh signaling in medulloblastoma

MB is the most common pediatric malignant brain tumor and is one of the leading causes of cancer-related mortality in children. These tumors arise either in the cerebellum or brainstem and have historically been classified based on histopathology. In 2010, an international consensus conference resulted in what is the current classification of MB into four distinct subgroups: WNT, SHH, Group 3 and Group 4 (Taylor et al., 2012). These subgroups are defined by their transcriptional profiles, and can be distinguished based on demographics, histology, DNA copy number aberrations, and clinical outcome.
Approximately 30% of all MB cases belong to the SHH subgroup. SHH-MB presents as the most common subgroup in infants (≤3 years old) and adults (≥18 years old). The role of Shh signaling in MB formation is in some ways not surprising given the mitogenic role of Shh signaling in normal cerebellum development. During postnatal day 3 to day 9 in mice, cerebellar granule neuron precursors (GNPs) undergo massive proliferation in response to paracrine Shh ligand secreted from underlying Purkinje cells. The GNPs migrate through the Purkinje layer to form the internal granule layer of the cerebellum, becoming unresponsive to Shh signals in the process and thus exiting the cell cycle to differentiate into mature granule neurons. However, if the Shh pathway is constitutively activated in GNPs, proliferation continues and can lead to formation of MB. It should be noted that non-SHH subgroup MBs are thought to have distinct cellular origins, including dorsal brain stem cells.

Transcriptome analyses and whole-genome sequencing have shown that among SHH subgroup MBs, there is quite a lot of heterogeneity (Kool et al., 2014; Northcott et al., 2011). PTCH1 mutations (~45%) and frequent loss of chromosome 9q are found in SHH-MB, whereas SMO mutations (~14%) are less common and are highly enriched in adult versus pediatric patients (Kool et al., 2014). SUFU mutations occur (~14%), with the majority of these found in infants (0-3 years old) (Kool et al., 2014). GLI2 amplifications (~8%) were identified in children ages 4-17 and predominantly co-occurred with TP53 mutations.

Subclassification of the MB subgroups can reveal important diagnostic factors. For instance, TP53 is one of the most frequently mutated genes in SHH-MB. Between ~10-20% of SHH-MB tumors are reported to have mutations in TP53 based on
sequencing large cohorts of patients (Kool et al., 2014; Zhukova et al., 2013). Moreover, it has been proposed that TP53 status is the most important risk factor for SHH tumors, with five-year overall survival reduced by almost half for patients with SHH/TP53 mutant tumors (Zhukova et al., 2013). A possible cause for the increased risk may be due to the high overall mutational rate in the presence of TP53 mutations (Kool et al., 2014).

Additionally, human TP53-mutated MB has been observed to undergo massive chromosomal rearrangements that occur in a one-step event, called chromothripsis, which is typically associated with genomic instability and poor survival in other cancers (Rausch et al., 2012).

Another important diagnostic factor in SHH-MB may be reduced activity of the tumor suppressor GNAS, which encodes the G protein Ga\(_s\) that can promote cAMP-dependent PKA activity. Gnas loss in murine cerebellar or brainstem progenitors induced formation of MBs with a SHH gene signature (He et al., 2014b). While this study attributed oncogenesis to reduced PKA activity, PKA-independent effects on Shh signaling may have also contributed to tumor formation. Importantly, GNAS mutations have been reported in human MB (Huh et al., 2014; Kool et al., 2014) and low expression of GNAS defines a particularly aggressive subset of SHH-MB (He et al., 2014b).

In addition to genetic mutations and copy number changes, epigenetic alterations can also play a role in MB. In a recent study comparing DNA methylation status, researchers identified hypo-methylation of VAV1, a gene for a GDP/GTP nucleotide exchange factor, as a frequent region-wide event in human SHH-MB compared to the other subgroups and normal cerebellum (Lindsey et al., 2015). The authors did not find
significant copy number alterations or mutations in this gene, but instead showed that hypomethylation was associated with elevated VAV1 expression. Silencing VAV1 with small interfering RNA in human MB cells significantly decreased their proliferation and resulted in G2 arrest and increased G0/sub-G1 cell fractions. Beyond this specific example, genomic data indicates a high prevalence of somatic alterations in chromatin-modifying genes of MBs, and thus suggests other epigenomic contributions to the underlying tumor biology (Batora et al., 2014).

The current standard of care for MB involves surgery, followed by craniospinal radiation and chemotherapy. This approach may cure up to 70-80% of patients. However, there is no standard effective treatment for recurrent tumors after radiation and prognosis following recurrence is poor. For young children, radiation is associated with long-term toxicities, including neurocognitive damage. Other side effects of radiation and chemotherapy include secondary malignancies and cardiopulmonary problems. Therefore, targeted therapies with reduced toxicity and improved efficacy are needed.

**Targeting Hh activation in tumors**

Hh signaling can be targeted at many levels, from blocking Hh ligands with antibodies to chemically inhibiting Gli function (Amakye et al., 2013). The largest and most clinically advanced group of Hh signaling inhibitors antagonizes the functions of Smo. In 2012, vismodegib (GDC-0449) was the first-in-class US Food and Drug Administration (FDA)-approved Smo inhibitor for the treatment of locally advanced, unresectable, and metastatic BCCs. In 2015, sonidegib (LDE225) was also approved
for locally advanced BCC. These and other Smo inhibitors are currently being evaluated in clinical trials for other cancers, including MB.

Phase I clinical trials with vismodegib for advanced solid tumors, including BCC and MB, offered initial indications that Smo inhibition was tolerable and in some cases effective for Hh-dependent tumors. These reports led to the ERIVANCE BCC phase II trial that secured vismodegib FDA approval (Sekulic et al., 2012). A 12-month follow-up to the primary ERIVANCE analysis was published last year, confirming efficacy and safety for vismodegib, with demonstrated durability of response (Sekulic et al., 2015). Also published last year was an interim analysis of the STEVIE trial, the largest vismodegib-treated patient series (499 patients) with advanced BCC reported to-date (Basset-Seguin et al., 2015). Based on the collective data, overall vismodegib response rates for locally advanced and metastatic BCC are ~45-70% and ~30-40%, respectively.

Common adverse events considered on-target for this class of drugs include muscle spasms, alopecia, and taste disturbance. Serious adverse events and treatment discontinuation due to adverse events are also reported. Furthermore, efficacy can be transient due to outgrowths of resistant tumors. Clinical trials with other Smo inhibitors, including sonidegib and IPI-926, consistently cite similar anti-tumor activity and toxicity profiles (Jimeno et al., 2013; Migden et al., 2015; Rodon et al., 2014).

Clinical trials with SHH-MB also indicate varied rates and durations of responses. A Phase I study of vismodegib in children with recurrent or refractory MB demonstrated complete but transient response in 1 out of 3 SHH-MB patients and no responses in any of the 13 non-SHH patients (Gajjar et al., 2013). Neither bone nor dental toxicity was detected, despite preclinical prediction of such events with Smo inhibitors in mice. In
follow-up Phase II trials, 43 adult and pediatric patients with recurrent MB from all four subgroups were treated with vismodegib (Robinson et al., 2015). Protocol-defined responses (3 adult and 1 pediatric) and radiographic responses (5 adult and 3 pediatric) were observed only for SHH-MB patients, and progression-free survival was longer in patients with SHH-MB versus those with non-SHH-MB. Unfortunately, all responses in SHH-MB patients were transient (~3-16 months before disease progression). Analysis of samples either pre- or post-treatment identified some likely causes of the observed resistance, including 3 patients with SUFU mutations and 3 patients with GLI2 amplifications. These initial clinical reports encourage further investigation of Hh-targeted therapies and, correspondingly, a better understanding of pathway regulation.

In the following chapters, we describe two complementary studies that aim to improve our understanding of and approaches toward targeted treatment of Shh-dependent MB. In the first study, we employed transposon mutagenesis in an in vitro model of Shh-dependent MB and found that genomic disruption of Oral-facial-digital syndrome 1 (Ofd1) can mediate resistance to Smo inhibition via loss of primary cilia. Independent, functional loss of other cilia components similarly causes drug resistance. Furthermore, genomic analysis of clinically resistant BCCs reveals mutations in primary cilia genes, including Ofd1. Strikingly, resistant cilia mutants are still dependent on active Shh signaling downstream of Smo. In the second study, we conducted a high-throughput chemical screen and identified several small molecules that reduce survival of Shh-dependent MB. Histone deacetylase (HDAC) inhibitors comprised a class of small molecules that blocked survival of both sensitive and Smo inhibitor resistant MB cells. Additionally, HDAC inhibitors reduced Shh signaling in vitro and tumor growth in
vivo. Together, our findings reveal a surprising mechanism of resistance to Smo targeted therapies and offer alternative strategies for treating resistant tumors.
CHAPTER 2: A TRANSPOSON MUTAGENESIS SCREEN IN MEDULLOBLASTOMA IDENTIFIES SUFU AND OFD1 AS SMO ANTAGONIST RESISTANCE GENES

Contributions:
Ekaterina Pak wrote chapter, prepared figures, and generated data for Figures 2.2-2.6 and Supplementary Figures S2.3-S2.5, S2.7.

Xuesong Zhao generated schematics for Figures 2.1 and 2.6 and data for Figures 2.2-2.4, 2.6-2.8, and Supplementary Figures S2.1, S2.2, S2.4-S2.7.

Kimberly Ornell generated data for Figures 2.2-2.4, 2.6-2.8, and Supplementary Figures S2.1, S2.2, S2.4-S2.7.

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Introduction

Resistance to targeted therapies, and particularly the clinically advanced Smo inhibitors, has received considerable attention in recent years (Metcalfe and de Sauvage, 2011). As described in Chapter 1, clinical trials have already demonstrated that not all patients with Shh pathway activated tumors respond to Smo inhibitors and even those who initially respond can develop resistance within months of treatment.

Reported mechanisms of resistance to targeted Hh pathway inhibition

The first report of a functionally characterized mechanism for clinically acquired resistance described a patient with widespread, metastatic medulloblastoma (MB) who initially showed dramatic tumor regression with vismodegib (Rudin et al., 2009). The patient rapidly relapsed due to a SMO mutation that abrogated drug binding (Yauch et al., 2009). Beyond this case and the clinical trials data summarized above, there is limited information on resistance in MB due to the low numbers of patients treated with Smo inhibitors to date. Although advanced basal cell carcinoma (BCC) is also rare, there is relatively more information on resistance for these patients.

Last year, two studies reported extensive genomic and functional analyses of clinically resistant BCCs. Together, they summarize several key concepts of Smo inhibitor resistance in BCC. First, reactivation of Shh signaling is the predominant form of resistance, solidifying BCC addiction to Shh signaling. This observation contrasts with other cancers that can hijack oncogenic signaling from multiple pathways. Second, the most common resistant mutations in BCCs involve the drug target SMO (~50-69% of resistant BCCs). Third, there are two classes of SMO mutations—those in proximity to
the SMO drug binding pocket (DBP) and those located distally. Finally, there are less frequent resistance mutations in other genes, including genes coding for regulatory units of the cAMP/PKA signaling axis, \( GLI2 \) amplification, and homozygous \( SUFU \) mutations. Again, these predominantly fall into the category of reactivating Shh signaling.

BCCs are recognized as one of the most mutated human cancers (Atwood et al., 2015). Despite the high mutation load, most BCC patients have a low rate (~20% per year) of acquired resistance to vismodegib. Given the strong dependence on Shh signaling in these tumors, the rates of acquired resistance may be limited by a confined repertoire of variants capable of conferring pathway maintenance in the presence of Smo antagonist. This hypothesis could also explain the observed high proportion of \( SMO \) mutations in resistant BCCs. Alternatively, since Smo inhibitors have only recently entered clinical practice and since BCC is a slow-growing cancer, it is possible that more patients may eventually acquire resistance and that new mechanisms of resistance will be revealed.

**Preclinical studies on resistance to targeted Hh pathway inhibition**

Corroborating and expanding on the clinical findings, there have been several studies on resistance in preclinical models of both BCC and MB. Early preclinical studies confirmed \( Smo \) mutations and \( GlI2 \) amplifications in murine models of MB resistance (Buonamici et al., 2010; Dijkgraaf et al., 2011). They also indicated that PI3K/AKT signaling, which regulates processes like cell growth and proliferation, is associated with resistance. Dijkgraaf et al. found that the PI3K/AKT pathway was active
in both sensitive and resistant tumors and that PI3K inhibition reduced, but did not block, tumor growth. Meanwhile, Buonamici et al. reported that addition of a PI3K inhibitor or a dual PI3K-mTOR inhibitor with the Smo inhibitor LDE225 delayed or prevented emergence of resistance. A follow-up study asked if preexisting mutations in *Pten*, a negative regulator in PI3K/AKT signaling, would alter the initial response to vismodegib (Metcalf et al., 2013). Surprisingly, vismodegib treatment restrained growth of *Pten*-deficient MB in the Hh-driven mouse model, but did not cause tumor regression. The resulting tumor stasis was distinct from the response seen in *Pten* wild-type MBs, which regressed with vismodegib treatment. Thus, although *Pten* loss does not cause resistance, the remnant tumors in this model have serious implications for long-term treatment durability. Importantly, these studies have clinical relevance since patients with SHH-MB harbor recurrent mutations in *PTEN*, *PIK3CA*, and *PIK3C2G* and analysis of SHH-MB tumors shows functional activation of PI3K signaling in >10% of samples (n=155) (Kool et al., 2014). Thus, combination or sequential treatment with Hh and PI3K inhibitors may improve prognosis for some SHH-MB patients.

We recently demonstrated that activation of the RAS/MAPK pathway can also cause resistance to Smo inhibitors (Zhao et al., 2015). Unlike previous reports that emphasized pathway addiction by reactivation of Shh signaling, RAS/MAPK-mediated resistance suppresses Shh signaling and instead shifts tumor cell dependence to MAPK/AKT signaling. We found that over-expression of mutant HRAS(G12V) or BRAF(V600E), as well as upstream activation by basic fibroblast growth factor (bFGF), induced resistance that could be reverted back to a sensitive phenotype after removing RAS/MAPK activation. RAS/MAPK-mediated resistance was characterized by increased
metastatic potential of the tumor cells. Notably, RAS/MAPK activation in a metastatic MB lesion and in three resistant BCC tumors provides clinical relevance for these findings.

Another type of resistance reported in SHH-MB is mediated by rare, quiescent Sox2+ cells that have the potential to re-populate the tumor via self-renewal and differentiation (Vanner et al., 2014). Expression of the Sox2 transcription factor had previously been associated with SHH-MB and shown to drive tumor cell proliferation (Ahlfeld et al., 2013). Sox2+ cell populations can be enriched following treatment with either Smo inhibitors or other anti-proliferative agents, emphasizing that intratumoral heterogeneity is yet another challenge for treating MBs.

**Mouse models of medulloblastoma**

In the Ptch+/− mouse model of Gorlin syndrome, some cerebellar granule neuron precursors (GNPs) continue to proliferate beyond the usual time frame, consequently resulting in formation of preneoplastic lesions that can develop into MB (Goodrich et al., 1997). About 14% of Ptch+/− mice develop MB by 6 months of age. Non-Gorlin syndrome mouse models include activation of Hh target genes in all GNPs by conditional loss-of-function of both Ptch1 alleles (Yang et al., 2008) or introduction of an activating SmoM2 or SmoA2 allele (Hatton et al., 2008). About 90-100% of mice harboring these mutations develop cerebellar lesions by 2 months.

*In vitro* systems for growing and maintaining Shh-dependent tumors have been more limited, since MB cells typically lose dependence on Shh signaling in a culture system (Sasai et al., 2006). Thus, most previous studies relied on either *in vivo* systems
or freshly isolated tumor cells that could be cultured for a short time before losing key characteristics of SHH-MB. We recently reported on a method for generating stable MB cell lines (SMB21, SMB55, and SMB56) that grow as neurospheres, are tumorigenic, and maintain key features of SHH-MB (Zhao et al., 2015). These cells were derived from Ptch\(^{+/−}\) mice that develop spontaneous MB. About 20% of tumors propagated in growth factor-free media were able to grow for many passages and were designated SMB lines. Importantly, SMB lines are dependent on Shh signaling and faithfully respond to Smo inhibitors, both by reduction in Gli1 mRNA and protein levels and cell survival. Additionally, the SMB21 and SMB55 cell lines had point mutations in p53, and all three SMB lines exhibited dysregulated p53 protein levels. As mentioned in Chapter 1, TP53 is one of the most frequently mutated genes in SHH-MB and thus our cell lines display several clinically relevant features. The cell line SMB21 will be the primary model system for both the transposon screen discussed in Chapters 2 and 3 as well as the drug screen described in Chapter 4.

**Results**

**Design of transposon-mediated mutagenesis screen**

As there have been several early reports of clinical resistance to Smo antagonists in BCC but very few examples in MB, we initiated preclinical studies on potential mechanisms of resistance in Shh-dependent MB. To identify candidate resistance genes, we launched a genome-wide transposon mutagenesis screen using the *piggyBac (PB)* transposon system, which is highly active in mammalian cells (Rad et al., 2010; Yusa et al., 2011). The transposon system used in this screen is composed of two components: the transposon, a piece of DNA that can integrate into the host
genome, and the transposase, an enzyme that mobilizes the transposon (Figure 2.1A). The transposon vector can either cause a loss-of-function phenotype by disrupting gene function or gain-of-function by promoter-mediated over-expression of genes adjacent to the transposon-inserted location. Both the transposon and transposase were introduced into SMB21 cells, an *in vitro* model of SHH-MB derived from Ptch*^{+/−}* mice (Zhao et al., 2015). One week after transfection, cells were plated in soft agar for selection in the Smo inhibitor LDE225. Individual resistant colonies developed after ~4-8 weeks and were isolated and propagated into resistant clone cell lines. These cell lines were re-tested by a multi-dose survival assay to confirm resistance. All clones were then sequenced to identify transposon insertion sites. A summary of the screen is illustrated in Figure 2.1B.
**Figure 2.1**

**A.**

Schematic diagram of the *piggyBac* transposase and transposon expression vectors used in the screen. The transposase vector contains a mouse codon-optimized version of the *PB* transposase coding sequence, controlled by the *CMV* promoter. The transposon vector has two expression cassettes. The first cassette contains GFP and puromycin resistance genes joined by a viral 2A sequence and driven by the *UbC* promoter. The second cassette contains a splice donor (SD) to promote over-expression of genes adjacent to the transposon-inserted location. The functional DNA cassette, which is flanked by FRT sites (blue arrows), is placed between a pair of *PB* terminal repeats (*PB* 5' and *PB* 3', black arrows).

**B.**

Summary of the screen to identify Smo inhibitor resistance genes.

---

**Figure 2.1 Design and overview of transposon mutagenesis screen. (A)** Schematic diagram of the *piggyBac* transposase and transposon expression vectors used in the screen. The transposase vector contains a mouse codon-optimized version of the *PB* transposase coding sequence, controlled by the *CMV* promoter. The transposon vector has two expression cassettes. The first cassette contains GFP and puromycin resistance genes joined by a viral 2A sequence and driven by the *UbC* promoter. The second cassette contains a splice donor (SD) to promote over-expression of genes adjacent to the transposon-inserted location. The functional DNA cassette, which is flanked by FRT sites (blue arrows), is placed between a pair of *PB* terminal repeats (*PB* 5’ and *PB* 3’, black arrows). **(B)** Summary of the screen to identify Smo inhibitor resistance genes.
Identification of transposon insertion sites reveals Sufu and Ofd1 as resistance gene candidates

From an initial screening of ~30 million cells, a total of 29 individual resistant clones were isolated, and 27 of these could be further propagated for detailed analyses. All 27 clones showed resistance in multi-dose secondary tests with LDE225 (Figure 2.2A,B). Resistance to Smo inhibition was further confirmed with several of the mutant clones by testing responses to the Smo inhibitors vismodegib and cyclopamine (Figure 2.2C,D).

Deep sequencing of PCR amplicons was used to identify transposon insertion sites for the resistant clones. The number of insertion sites per clone was ~11 transposons on average (range of 2-73) (Table 2.1). Some resistant clones had unique transposon insertion sites in the same genes, which we called recurrent insertions (Table 2.2). The two genes with the greatest number of recurrent transposon insertion sites were Sufu (11 unique insertions in 13 clones) and Ofd1 (2 unique insertions in 3 clones) (Figure 2.3A,B). Loss of Sufu or Ofd1 protein in these clones was confirmed by immunoblot (Figure 2.3C,D). Identification of Sufu as a candidate resistance gene validated the screening methodology, since Sufu is a known negative regulator of Hh signaling and functional loss of Sufu has previously been shown to cause drug resistance (Kool et al., 2014; Lee et al., 2007; Zhao et al., 2015). One possible explanation for the large number of Sufu hits could be genetic copy number loss of Sufu during the screen. In fact, we found that the majority of Sufu mutant clones had reduced Sufu gene copy number (Supplementary Figure S2.1). In contrast, we did not observe
many copy number changes for Ccnd1, Gli2, or Mycn, three other genes that are recognized as mediating resistance to Smo antagonists.
Figure 2.2 Cell lines with transposon insertions are resistant to multiple Smo inhibitors. (A) A total of 27 resistant clones that were propagated in culture after selection in LDE225 show resistance by an MTS-based secondary screen with 3 doses of LDE225. SMO(D477G) cells are a positive control previously reported to show resistance to Smo inhibitors. The heatmap represents the mean of 3 independent experiments. (B-D) Representative resistant clones from the transposon screen display multi-dose resistance to the Smo inhibitors LDE225 (B), vismodegib (C), and cyclopamine (D) compared to parental cells (n = 3-4 independent experiments, mean ± SEM). Gli2ΔN cells express a constitutively active form of human GLI2 and are a positive control for resistance.
Figure 2.2 (continued)

A.

B.

C.

D.
Table 2.1 Number of transposon insertions in each resistant clone.

<table>
<thead>
<tr>
<th>Resistant Clone</th>
<th>Number of Transposon Insertions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB5</td>
<td>4</td>
</tr>
<tr>
<td>DB6</td>
<td>2</td>
</tr>
<tr>
<td>DB9</td>
<td>16</td>
</tr>
<tr>
<td>DC2</td>
<td>20</td>
</tr>
<tr>
<td>DC3</td>
<td>15</td>
</tr>
<tr>
<td>DC9</td>
<td>18</td>
</tr>
<tr>
<td>DD2</td>
<td>16</td>
</tr>
<tr>
<td>DE9</td>
<td>6</td>
</tr>
<tr>
<td>DF2</td>
<td>12</td>
</tr>
<tr>
<td>DF3</td>
<td>6</td>
</tr>
<tr>
<td>DF4</td>
<td>9</td>
</tr>
<tr>
<td>DG2</td>
<td>15</td>
</tr>
<tr>
<td>DG8</td>
<td>3</td>
</tr>
<tr>
<td>GD10</td>
<td>12</td>
</tr>
<tr>
<td>GE8</td>
<td>12</td>
</tr>
<tr>
<td>GG6</td>
<td>14</td>
</tr>
<tr>
<td>HD4</td>
<td>5</td>
</tr>
<tr>
<td>HD5</td>
<td>7</td>
</tr>
<tr>
<td>HF2</td>
<td>11</td>
</tr>
<tr>
<td>HF3</td>
<td>7</td>
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<td>LF2</td>
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</tr>
<tr>
<td>LH2</td>
<td>16</td>
</tr>
</tbody>
</table>
Table 2.2 Genes with recurrent transposon insertions are top hits in the resistance screen. Transposon insertions were considered recurrent if different insertion sites in the same gene (# of Unique Insertions) were identified in separate clones (# of Clones). As depicted in Figure 2.3, the Sufu gene had 2 locations where transposons inserted in the same site of different resistant clones (identical insertion locations were found in clones DE9 and DC2, as well as clones DG8 and DF3). The Ofd1 gene had one identical insertion site found in resistant clones GD10 and GG6. The p-value represents the probability of finding the given number of unique insertions in the corresponding gene by chance with no selective pressure, based on the following: (1) total number of transposon insertions in the screen, (2) number of insertions in the given gene, and (3) gene size.

<table>
<thead>
<tr>
<th>Gene</th>
<th># of Clones</th>
<th># of Unique Insertions</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sufu</td>
<td>13</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Ofd1</td>
<td>3</td>
<td>2</td>
<td>1.61E-05</td>
</tr>
<tr>
<td>Rgl1</td>
<td>2</td>
<td>2</td>
<td>7.23E-05</td>
</tr>
<tr>
<td>Fat3</td>
<td>2</td>
<td>2</td>
<td>0.001333</td>
</tr>
</tbody>
</table>
Figure 2.3

**A. Sufu**

\[
\text{DG2 \quad HF2 \quad DE9 \quad HD5 \quad GE8 \quad DB5 \quad DC3 \quad DB9 \quad DF2 \quad DG8 \quad DF3 \quad DD2}
\]

10 kb

**B. Ofd1**

\[
\text{GD10 \quad GG6 \quad LE2}
\]

10 kb

**C.**

<table>
<thead>
<tr>
<th></th>
<th>Parental</th>
<th>DB5</th>
<th>DF2</th>
<th>DF3</th>
<th>DC2</th>
<th>HDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sufu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**D.**

<table>
<thead>
<tr>
<th></th>
<th>Parental</th>
<th>LE2</th>
<th>GD10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ofd1</td>
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</tr>
<tr>
<td>Actin</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Figure 2.3 Sufu and Ofd1 are the top screen hits with multiple transposon insertions.** (A) Next-gen sequencing identified 13 resistant clones with transposon insertions in Sufu. The direction of transposon insertion is indicated by blue flags (transposon promoter faces endogenous Sufu gene promoter) and red flags (transposon promoter is in the opposite direction of the Sufu promoter). Overlapping flags indicate identical transposon sites and may indicate clones that arose from the same initial resistance event. (B) A total of 3 resistant clones had transposon insertions in Ofd1, two of which may have originated from the same initial resistance event (GD10 and GG6). (C) Resistant clones with transposon insertions in Sufu have lost Sufu protein. (D) Resistant clones with transposons in Ofd1 have lost Ofd1 protein.
Sufu and Ofd1 loss correlates with active Shh signaling in tumor cells but precludes ligand-induced Shh activation in non-tumor cells

Pathway reactivation is a common mechanism of clinical resistance to Smo antagonists. To confirm that resistant clones with Sufu loss had active Shh signaling, and to assess pathway status in clones that lost Ofd1, we analyzed Gli1 levels, which are a readout for Shh pathway transcriptional activation.

Both Sufu and Ofd1 mutant cells displayed activation of Shh signaling based on Gli1 protein and mRNA levels (Figure 2.4A,B). Although Gli1 levels in Ofd1 resistant clone LE2 were lower compared to the other mutants and untreated parental cells, both protein and mRNA levels were greater than the off state seen in parental cells treated with LDE225. Furthermore, RNAseq indicated that there were no consistent changes in Shh target gene expression between parental cells and Sufu and Ofd1 mutant cells (Supplementary Figure S2.2). In contrast, treating parental cells with LDE225 altered the pattern of gene expression, highlighting the contrast between active and repressed Shh pathway activity. Observing that Shh signaling is active in Sufu and Ofd1 resistant clones suggests that resistance is mediated by reactivation of Shh signaling downstream of Smo.

To determine if loss of Sufu or Ofd1 function also correlates with active Shh signaling in non-tumor cells, we employed lentiviral CRISPR/Cas9 to specifically target the Sufu or Ofd1 locus in Shh Light II (SL2) cells and mouse embryonic fibroblasts (MEFs). The SL2 cells are derived from NIH 3T3 cells stably expressing a Gli-dependent firefly luciferase reporter gene and a constitutive Renilla luciferase gene (Taipale et al., 2000). Stimulation of SL2 cells by SAG, a small molecule Smo agonist,
Figure 2.4

Figure 2.4 Sufu and Ofd1 resistant clones have active Shh signaling. (A) Resistant clones with Ofd1 loss maintain active Shh signaling as indicated by Gli1 protein, which is not reduced after 24 hours of LDE225 treatment (0, 10, 100, and 1000 nM). Clones with Sufu loss also maintain active Shh signaling, as expected. (B) Sufu and Ofd1 mutants also express Gli1 mRNA that is not reduced by treatment with LDE225. Gli1 mRNA is normalized to GAPDH levels and also normalized to parental levels from the same experiment. Two-way ANOVA with Dunnett’s correction, ***p<0.001. Statistics are only shown for parental and GD10 cells (n=3 independent experiments for parental and GD10 cells, mean ± SEM; n=1 for all other cell lines).
(Chen et al., 2002) increases the Firefly/Renilla ratio, serving as readout for pathway activation. As expected, depletion of the negative pathway regulator Sufu resulted in constitutive, ligand-independent pathway activation (Figure 2.5). However, depletion of Ofd1 resulted in a low level of pathway activity that could not be stimulated by SAG. Similarly, Ofd1 depletion in wild-type MEFs resulted in low levels of Gli1 mRNA that also could not be stimulated by SAG (Supplementary Figure S2.3A). We tested the consequences of Ofd1 depletion in a variety of mutant MEFs lines with homozygous deletions in genes that can affect Hh activity (Supplementary Figure S2.3). In all non-tumor cell lines tested, we found that Ofd1 depletion maintains low Shh target gene expression and prevents SAG-induced pathway activation.

**Validation of Ofd1 and Sufu as resistance genes**

To unequivocally validate Sufu and Ofd1 as true resistance genes, we took three separate approaches. First, we took advantage of the transposon design, in which FRT sites flank the DNA cargo (Figure 2.6A). Lentiviral delivery of a mouse codon-optimized FLP recombinase (Flp) to the resistant clones can cleave the FRT sites, thus removing the majority of the transposon(s). By this approach, it is possible to revert mutants back to an LDE225-sensitive phenotype and demonstrate a direct correlation between transposon insertions and resistance. A successful rescue for drug sensitivity by transposon removal reduces the likelihood that resistance is caused by a background mutation.

Among the 3 Ofd1 mutant clones, we observed a rescue for LDE225 sensitivity in clone LE2 (Figure 2.6B). Importantly, clone LE2 had a transposon insertion in an intron
Figure 2.5

A. CRISPR/Cas9-mediated depletion of Ofd1 or Sufu reduces protein levels of these targets in SL2 cells. (B) Depletion of Ofd1 in SL2 cells abrogates SAG-induced activation of Shh signaling. Student's t-test, *p<0.05 (n=3 independent experiments, mean ± SEM).
of the *Ofd1* gene, whereas clones GD10 and GG6 had insertions in an exon and did not rescue for sensitivity (Figure 2.3B, Supplemental Figure S2.4). It is possible that the footprint left behind by the transposon after Flp rescue is sufficient to disrupt gene function. The Flp-rescued LE2 cells re-expressed Ofd1 protein and mRNA, and displayed LDE225-induced pathway inhibition measured by reduction in Gli1 levels (Figure 2.6C-E). The *Sufu* mutant clone DB5 could also be rescued by Flp addition, showing sensitivity to LDE225 and re-expression of Sufu protein (Figure 2.6F,G). We comprehensively tested 27 out of the 29 resistant mutants from the transposon screen for ability to rescue by Flp and successfully rescued LDE225 sensitivity in 10 of these clones (Supplementary Figure S2.5). Screen mutants with transposon insertions outside of *Sufu* or *Ofd1* that can also be rescued by Flp are excellent starting points for identification of other resistance genes.
Figure 2.6 Resistant clones can be rescued for drug sensitivity by removal of transposons. (A) Schematic for removal of the transposon DNA cargo by Flp/FRT-mediated DNA recombination. (B) Resistant clone LE2 (transposon in Ofd1) can be rescued for sensitivity to LDE225 (LDE) by lentiviral-delivered Flp. Relative survival was assessed at 0, 10, 100 and 1000 nM LDE. Control infection with tdTomato (Tomato) did not rescue for sensitivity. One-way ANOVA with Dunnett’s correction for each treatment condition versus 0 nM LDE, *p<0.05, **p≤0.005, ***p<0.0005 (n=3 independent experiments, mean ± SEM). (C,D) Addition of Flp to clone LE2 re-expresses Ofd1 protein (C) and mRNA (D). Ofd1 mRNA was normalized to GAPDH levels. Shh pathway inhibition does not change Ofd1 mRNA levels. Two-way ANOVA with Tukey correction, *p<0.05 for LE2(Tomato) groups versus parental with LDE and LE2(Flp) with DMSO, ++p<0.01 for LE2(Tomato) groups versus LE2(Flp) with LDE (n=3 independent experiments, mean ± SEM). Flp also rescues Shh pathway inhibition with LDE as measured by Gli1 protein (C) and mRNA (E). Gli1 mRNA was normalized to GAPDH levels. T-test, **p<0.01 (n=3 independent experiments, mean ± SEM). (F) Resistant clone DB5 (transposon insertion in Sufu) can also be rescued for sensitivity by Flp. ++p<0.0001 (n=3 independent experiments, mean ± SEM). (G) Flp addition to clone DB5 re-expresses Sufu protein and rescues Shh pathway inhibition by LDE.
In the second approach to validate our candidate resistance genes, we expressed full-length *Sufu* or *Ofd1* in the mutant clones and confirmed rescue of LDE225 sensitivity by assaying Gli1 levels and cell survival (Figure 2.7). Finally, independent CRISPR/Cas9-mediated depletion of *Sufu* or *Ofd1* in parental cells recapitulated the resistance phenotype observed in the screen clones (Figure 2.8). To corroborate that resistance due to loss of *Sufu* or *Ofd1* can occur in other MB cells, we tested and confirmed that depletion of either target caused resistance in two independently established cell lines (Supplementary Figure S2.6).

In summary, we completed a forward genetic screen for candidate Smo inhibitor resistance genes in Shh-dependent MB. We chose to focus on *Sufu* and *Ofd1* because they were the top two recurrently mutated genes. We validated that both genes can cause resistance by three different methods: removal of the transposon, rescue by over-expression, and CRISPR/Cas9-mediated depletion. Importantly, both *Sufu* and *Ofd1* resistant mutants displayed activation of the Shh pathway. A summary of the screen and follow-up studies described is presented in Supplementary Figure S2.7.
Figure 2.7 Exogenous Ofd1 and Sufu rescue transposon mutant sensitivity to Smo inhibition. (A) Lentiviral-delivered Ofd1 (LV-Ofd1) in the Ofd1 mutant clone GG6 re-expresses Ofd1 protein and rescues sensitivity to LDE225 (LDE) as measured by Gli1 levels. Cells were treated with 1 µM LDE for 24 hours. Control infection with lentivirus expressing tdTomato (Tomato) did not rescue sensitivity. (B) Expression of Tomato and Ofd1 in parental cells does not alter functional sensitivity to LDE. Relative survival was assessed at 0, 10, 100 and 1000 nM LDE. (C) Expression of Ofd1 in clone GG6 rescues sensitivity to LDE. (D,E) Expression of Sufu in the Sufu mutant clones DB5 and DG8 also rescues sensitivity to LDE. Statistical analysis by one-way ANOVA with Dunnett’s correction for each treatment condition versus 0 nM LDE, *p<0.05, **p<0.005, ***p≤0.0005, +++p<0.0001 (n = 3-4 independent experiments, mean ± SEM).
Figure 2.7 (continued)

A.

<table>
<thead>
<tr>
<th></th>
<th>Parental LV-Tomato</th>
<th>GG6 LV-Tomato</th>
<th>GG6 LV-Ofd1</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDE -</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LDE +</td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

Gli1

Ofd1

Actin

B.

C.

D.

E.

n.s.

n.s.

% Survival

% Survival

% Survival

% Survival
Figure 2.8 Depletion of Ofd1 and Sufu causes resistance in parental cells. (A)

CRISPR/Cas9-mediated depletion of Ofd1 reduces Ofd1 protein levels and abolishes pathway inhibition as measured by Gli1 protein in SMB21 cells. (B) Ofd1 depletion results in functional resistance to LDE225 (LDE). Relative survival was assessed at 0, 10, 100 and 1000 nM LDE. (C) Depletion of Sufu reduces Sufu protein levels. (D) Sufu depletion results in functional resistance to LDE. Statistical analysis by one-way ANOVA with Dunnett’s correction for each treatment condition versus 0 nM LDE, *p<0.05, ***p<0.0005, ++p<0.0001 (n = 3 independent experiments, mean ± SEM).
Discussion

Transposon mutagenesis for identification of resistance mutations

There are several methods that can be used to screen for resistance to targeted inhibitors in mammalian cells. These include testing candidate genes individually and using libraries for knock-down or over-expression. Additionally, increasing or intermittent dosing schedules can stimulate the emergence of resistance. We chose transposon mutagenesis as our approach based on the key features described here.

Transposon mutagenesis has been a successful technique for recent studies on MB. For example, the Sleeping Beauty (SB) transposon system has been used in Ptch+/− mice to study tumor initiation (Genovesi et al., 2013), metastasis (Wu et al., 2012), and recurrent mutations following surgery and radiation (Morrissy et al., 2016). Sleeping Beauty was the first active DNA transposon generated for use in mammalian cells (Ivics et al., 1997). In the nearly two decades since it was first discovered, other transposons like piggyBac, have been reported. The piggyBac transposon system characteristically has efficient transposition in mammalian cells, large cargo capacity, and different integration preferences compared to Sleeping Beauty (Rad et al., 2010; Yusa et al., 2011).

There are also several technical advantages of transposon-based mutagenesis. Since mutations are tagged by the transposon DNA, we can rapidly identify the locus responsible for an observed phenotype. By re-introducing transposase into the same cell, or by using Flp to remove the functional portion of the transposon from the inserted locus, we can reverse the induced mutation back to wild-type and thus verify the relationship between transposon mutations and the phenotypes they cause. Finally, the
DNA cargo placed between the transposon inverted repeats can be designed for specific functions such as expressing selection markers and promoters that can drive over-expression of mutated genes for simultaneously screening both gain- and loss- of function changes.

Transposon screen results

Here, we report validation of our chosen mutagenesis system, and show that it can be used to faithfully identify candidate resistance genes. The most frequent hit in the screen was transposon integration in Sufu and resulting loss of the protein product. Identifying Sufu as a hit addresses three major concerns regarding use of the PB transposon system. First, this approach works for loss-of-function mutations. Second, the screen identifies mutations that reactivate the pathway downstream of Smo. Third, we can rapidly move from new resistant clones to hit identification. Nonetheless, we recognize that this approach is unconventional. A limitation of this method is that the location of transposon integration may be in the midst of several genes with overlapping genomic DNA. Thus, we followed-up candidate hits with several validation methods, including rescue and independent protein depletion tests.

It is likely that the transposon screen selected predominantly more loss-of-function than gain-of-function mutants. The possible regions of integration for a gain-of-function mutation are more limited compared to the available genomic loci that can be disrupted to result in loss-of-function. However, we did validate that over-expression can occur and can be rescued by removal of the transposon (data not shown).
Additionally, the screen likely selects for genes that are haploinsufficient, since the second copy of a gene undisrupted by a transposon may be sufficient to compensate for the loss of one allele. Importantly, both the human and mouse \textit{OFD1} genes are located on the X-chromosome. The transposon screen was conducted on MB cells that were derived from male mice, and thus disruption of one allele was sufficient to disrupt all Ofd1 functions in the cells.

**Tumor cell genetic background may mediate reactivation of Shh signaling after Ofd1 loss**

CRISPR/Cas9-mediated depletion of Ofd1 in non-tumor cells abrogates ligand-induced pathway activation. Given that our SMB21 cells have known genetic changes (\textit{Ptch1} loss and \textit{p53} mutation) that can affect Shh signaling, we also tested mutant MEFs with these changes. Ofd1 depletion in p53\(^{+/−}\) MEFs suppressed ligand-induced stimulation just like in wild-type MEFs (Supplementary Figures S2.3). Loss of response to SAG stimulation was also seen in Gli2\(^{−/−}\) MEFs, albeit at a suppressed overall pathway activity due to loss of the activator functions of Gli2. However, in Ptch\(^{−/−}\) MEFs, we found an additional effect of Ofd1 depletion. Ptch\(^{−/−}\) MEFs have increased basal activation of Shh signaling. Ofd1 loss in Ptch\(^{−/−}\) MEFs not only abrogates stimulation by SAG, but also suppresses the basal pathway activity in these cells. We extended our analysis to Gli3\(^{−/−}\) MEFs, which also have some basal Hh activity due to loss of negative regulation by Gli3R. Ofd1 depletion in Gli3\(^{−/−}\) MEFs prevented SAG-induced pathway stimulation, like in all other MEFs lines. However, Ofd1 depletion did not reduce the basal pathway activation in Gli3\(^{−/−}\) MEFs like it did in Ptch\(^{−/−}\) MEFs. These results
highlight the importance of genetic background in predicting whether there will be reactivation of Shh signaling (and possibly resistance) in the context of cilia loss. Based on these observations, it is possible that the different levels of Shh signaling in Ofd1 resistant clones from the screen (LE2, GD10, and GG6) (Figure 2.4) may be explained by Sufu copy number loss in GD10 and GG6 cells (Supplementary Figure S2.1). We are currently testing if Ofd1 loss in Sufu+/- MEFs raises the basal level of Shh signaling above wild-type MEFs. These studies will provide further information on how genetic background is an important factor in Shh pathway reactivation and resistance.

Conclusion

In this chapter, we present the application of a genome-wide transposon mutagenesis screen for identification of candidate resistance genes to Smo antagonists. We validate candidate resistance genes with follow-up rescue and CRISPR/Cas9-mediated depletion tests. We also show that resistant mutants reactivate Shh signaling and that the level of signaling may depend on the genetic background of mutant cells. Follow-up studies presented in Chapter 3 focus on further exploring the mechanisms by which Ofd1 functional loss causes resistance.

Methods

Animals

All experimental procedures were done in accordance with the National Institutes of Health guidelines and were approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee. Pcth+/- mice (Goodrich et al., 1997) (Jackson Laboratory).
**SMB cell culture**

SMB cell lines (SMB21, SMB55, SMB56) were derived from spontaneous MB tumors in *Ptch*<sup>+/−</sup> mice (Zhao et al., 2015). Briefly, MB tumors were dissected and dissociated with accutase. Dissociated cells were cultured as neurospheres at 37°C in a humidified incubator with 5% CO<sub>2</sub> in DMEM/F12 media (2% B27, 1% penicillin/streptomycin). To passage cultures, cells were dissociated with accutase and plated 1:3 in fresh media.

**Plasmids**

The *mPBase* plasmid was obtained from the Wellcome Trust Sanger Institute (Cadinanos and Bradley, 2007). The *piggyBac* (*PB*) transposon screen vector was constructed as follows. The *PB* backbone was gene synthesized, including the *piggyBac* 5’ and 3’ terminal repeats linked by two FRT sites and multiple cloning sites. The *CMV* promoter expression cassette was cloned into the *PB* backbone vector from a pcDNA3.1 vector (Invitrogen). The *ubiquitin C* (*UbC*) promoter expression cassette was cloned from a *pPB-UbC* plasmid (Yusa et al., 2009). The *GFP-2A-PTK* fragment (for GFP expression and puromycin selection) was cloned into the *UbC* expression cassette. For lentiviral expression constructs, coding sequences of *EGFP* (Clontech), *tdTomato* (Clontech), *FLPo* (Addgene plasmid 13793) (Raymond and Soriano, 2007), mouse *Sufu*, mouse *Ofd1*, and *Luc2* (Promega) were subcloned into the Gateway-compatible lentiviral vector pLX304 (Addgene plasmid 25890) (Yang et al., 2011). LentiCRISPR was a gift from Feng Zhang (Addgene plasmid 49535). pLKO.1 lentiviral
shRNAs were from the Broad Institute RNAi Consortium (TRC). All plasmids were verified by sequencing.

**Establishing SMB stable lines with the piggyBac transposon**

For transposon mutagenesis and for stable expression of individual genes, $1 \times 10^6$ SMB cells were transiently transfected using 5 µg of *mPBase* plasmid, 5 µg of *PB* transposon plasmid, and 30 µL of Fugene6 (Roche). For mutagenesis, the *PB* transposon plasmid had a splice donor following a CMV promoter, as illustrated in Figure 2.1A. For expression of specific constructs, such as Gli2ΔN, the gene of interest was downstream of the CMV promoter. For target gene expression, selection with puromycin (1 µg/mL) was initiated 48 hours post transfection, and continued for 3 weeks. Stable expression of target genes was confirmed by immunoblots.

**Primary transposon mutagenesis screen in soft agar**

1 week after transfection of *mPBase* and *PB* transposon screen vectors, 30 million SMB21 cells were plated in soft agar. After 4-8 weeks of selection in 1µg/mL LDE225, 29 individual resistant clones were isolated. 27 clone cell lines were successfully established and propagated.

**Cell survival assays with pharmacologic inhibition**

SMB cells were seeded in 96-well plates (3 x $10^4$ cells per well). Serial dilutions of the relevant compound in DMSO were used, yielding final drug concentrations ranging from 10 µM to 0.01 nM. In all cases, the final volume of DMSO did not exceed 1%. Cells
were incubated for 72 hours following addition of LDE225, vismodegib or cycloponamine. Cell viability was measured using CellTiter 96 Aqueous One Solution (Promega), and calculated as a percentage of control (DMSO-treated cells). A minimum of three replicates was performed for each cell line and drug combination. Survival curves were modeled using a nonlinear regression curve fit with a sigmoid dose–response, and displayed using GraphPad Prism 6. 10 mM stocks of compounds were made in DMSO and stored at -20°C. LDE225, vismodegib (GDC-0449), and cycloponamine were purchased from Selleck Chemicals.

**Transposon insertion identification**

Genomic DNA from each clone was extracted using DNeasy Blood & Tissue Kit (Qiagen 69504). 0.5 µg of DNA was digested with Sau3AI restriction enzyme, then ligated with a splinkerette adaptor. To amplify the junction fragments between the flanking genomic sequence and the transposon insertion, two rounds of PCR reaction were used with nested-PCR primers. A third round of PCR was used to add a barcoded Illumina sequencing adaptor. PCR products from individual clones were pooled together and purified with QIAquick PCR purification Kit (Qiagen). The resulting purified PCR product library was then sequenced using Nextgen sequencing (Illumina MiSeq) by the Molecular Biology Core Facilities at Dana-Farber Cancer Institute. Raw sequences were first de-multiplexed according to barcodes, and low QC reads were discarded. The splinkerette adapter sequence and *piggyBac* transposon terminal repeat sequences were trimmed off. Processed reads were then mapped to the mouse genome using the
bowtie algorithm. The site and orientation of transposon insertion was determined from mapped sequences.

**Rescue by transposon cargo removal with lentiviral-delivered FLPo**

Transposon cargo removal was achieved using lentiviral-delivered FLPo to excise the two FRT sites flanking the transposon cargo. FLPo-FRT recombination also removed a puromycin selection cassette, rendering cells sensitive to puromycin. For transposon cargo removal, 0.5 x10⁶ SMB cells were infected with 1 mL of viral supernatant of LV-tdTomato or LV-FLPo. 48 hours after infection, blasticidin (2 µg/mL) was added, and cells were selected for 3 weeks.

**Lentiviral production**

Lentivirus was generated using the protocol from The RNAi Consortium (TRC) at the RNAi Platform of the Broad Institute of MIT and Harvard at [http://www.broadinstitute.org/rnai/public/](http://www.broadinstitute.org/rnai/public/). Briefly, 293T cells (ATCC) were seeded in 6-well plates and transfected with 1 µg of lentiviral plasmid, 900 ng Δ8.9 (gag, pol), and 100 ng VSV-G using 6 µL Fugene6 transfection reagent (Roche). Viral supernatants were harvested at 48 hours and 72 hours post transfection in DMEM/F12 media.

**CRISPR/Cas9-mediated depletion**

Targeted depletion of Ofd1 or Sufu was achieved using the lentiCRISPR vector system (Shalem et al., 2014). Infected cells were selected with 1µg/ml puromycin for 2-4 weeks before downstream experiments.
**Immunoblotting**

Cell lysates were made in RIPA buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM DTT, 10 mM NaF, 1 mM NaVO$_3$, 1 mM PMSF) with protease inhibitor cocktail (Roche). Lysates were quantified (Bradford assay), normalized, reduced, denatured (95°C) and resolved by SDS gel electrophoresis on 4-12% Bis-Tris gels (Invitrogen). Proteins were transferred to PVDF membranes (Biorad) and probed with primary antibodies recognizing Gli1 (Cell Signaling, 1:1000), Sufu (Cell Signaling, 1:1000), Actin (Sigma, 1:10,000), Gli2 (Aviva, 1:1000), V5 tag (Invitrogen, 1:1000). Ofd1 antibody was a generous gift from Jeremy Reiter. Secondary antibodies (goat anti-mouse or anti-rabbit) are HRP-linked (Biorad). Proteins were visualized using film or ImageQuant LAS 4000 imager.

**Quantitative RT-PCR**

RNA was extracted from cells using Trizol (Invitrogen) and RNeasy kit (Qiagen) according to the manufacturer’s protocol. Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s specifications. Quantitative real-time PCR was performed using TaqMan gene expression assays (Applied Biosystems) to assess expression of Gli1 (Mm00494645), Gli2 (Mm01293117), Sufu (Mm00489385), and Ofd1 (Mm00616877_m1). Each analysis was performed in triplicate. Expression was normalized to Gapdh (4352339E).
Gene copy number analysis

Genomic DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen). Genomic copy number for Sufu was determined by quantitative real-time PCR reagents (Applied Biosystems) with custom-designed primers using 5 ng of genomic DNA per reaction. Sufu forward primer: GCTTACCCAGAAGGCAGAGG; Sufu reverse primer: ACAGCGTATGTTCACCACCA; SINE1 forward primer: AGATGGGCTGAGTGGGTAAGG; SINE1 reverse primer: GTGGAGGTCAGAGGACAAAATT). Real-time PCR was carried out with the cycling method (50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute) using 5 ng of genomic DNA per reaction. Copy number was calculated as 2(T DNA/C DNA), where T DNA and C DNA are the calculated amounts of test gene DNA at the recorded C t for tumor and calibrator, respectively. Copy number data for Sufu were normalized to SINE1 elements with the formula 2(T DNA/L dnaT)/(C DNA/L dnaC), where T dna and C dna represent the amounts of Sufu-calculated DNA in the tumor and calibrator, respectively, and L dnaT and L dnaC are the corresponding amounts of their SINE1 DNA at the recorded C t values.

RNAseq analysis

RNA was isolated from cells, primary tumors and normal mouse brain using RNeasy Kit (Qiagen). Purified RNA samples were processed and applied to Affymetrix Mouse Genome 430A 2.0 Array at the DFCl microarray facility according to standard protocol (http://macf-web.dfci.harvard.edu/). Arrays were analyzed using GenePattern software (http://www.broadinstitute.org/cancer/software/geneexpression) (Reich et al.,
SL2 cell culture and luciferase assay

SL2 cells (ATCC Catalog # CRL-2795) were plated in 96-well opaque tissue culture dishes (Costar Catalog # 3917) and maintained in growth media (DMEM, 10% calf serum, 1% penicillin/streptomycin, 0.4 mg/mL G418, and 0.15 mg/mL zeocin). In SL2 stimulation experiments, growth media was removed and cells were stimulated with 300 ng/ml SAG or the same volume of vehicle (1:1 DMSO to PBS) in low serum conditions (DMEM, 0.5% calf serum, 5mM HEPES). After 3 days of stimulation, firefly and Renilla luminescences were measured with the Dual-Glo Luciferase Assay System (Promega Catalog # E2920), according to the manufacturer’s protocol.

MEF cell culture and stimulation

Gli3−/− and Ptch−/− MEFs were a generous gift from Matthew Scott (Stanford University School of Medicine, Stanford, CA). P53−/− MEFs were a generous gift from James DeCaprio (Harvard University, Boston, MA) (Poulin et al., 2004). Wild-type and Gli2−/− MEFs were generated as previously described (Eisner et al., 2015). Cells were cultured at 37°C in a humidified incubator with 5% CO₂ in DMEM media (10% heat-inactivated FBS, 1% L-glutamine, 1% penicillin/streptomycin). To passage cultures, cells were dissociated with 0.25% Trypsin-EDTA and plated 1:2 in fresh media.
Statistical analysis

Statistical analyses were performed with the Student’s t test, one-way ANOVA, or two-way ANOVA with Dunnett’s or Tukey test, as indicated. A p value of 0.05 or less was considered statistically significant. All data analyses were performed using Microsoft Excel or GraphPad Prism 6.
CHAPTER 3: OFD1 MUTATION AND OTHER MECHANISMS OF PRIMARY CILIA LOSS CAUSE RESISTANCE TO SMO ANTAGONISTS IN SHH-DEPENDENT TUMORS

Contributions:
Ekaterina Pak wrote chapter, prepared figures, and generated data for Figures 3.1, 3.4, 3.5 and Supplementary Figures S3.2, S3.3.

Xuesong Zhao generated data for Figures 3.2-3.5 and Supplementary Figures S3.1.

Kimberly Ornell generated data for Figures 3.2 and 3.3.

Ethan MacKenzie generated data for Figure 3.1

Acknowledgements:
Thank you to Maria Ericsson at the Harvard Medical School Electron Microscopy Facility for helping collect images.
Introduction

Primary cilia in Hh-dependent tumors

As described in Chapter 1, primary cilia can both promote and oppose Hh signaling. Pathway activation involves translocation of Smo and the Sufu-Gli complex into the cilium, where the latter dissociates so that activated Gli can go to the nucleus and transcribe target genes. Primary cilia are also thought to be necessary for processing full-length Gli (GliFL) proteins into repressor forms that turn off target genes. The roles of primary cilia in Hh-dependent tumors can reflect this duality. Two back-to-back studies revealed that primary cilia can either promote or inhibit Shh-driven tumorigenesis, depending on whether the pathway is activated upstream or downstream of cilia function (Han et al., 2009; Wong et al., 2009). Specifically, cilia loss blocks tumor formation driven by a constitutively active Smo (SmoM2). In contrast, removal of primary cilia in the context of a constitutively active GLI2 (GLI2ΔN) accelerates tumor formation. In part, this difference may occur because cilia are required not only for Smo function but also for Gli3 repressor (Gli3R) formation. Thus, removing cilia in mice expressing Gli2ΔN would remove the inhibitory roles of Gli3R and help drive tumorigenesis. These results were observed for both basal cell carcinoma (BCC) and medulloblastoma (MB) formation.

An extension of these findings was more recently reported for Ptch+/− models of MB, in which cilia were not only necessary for tumor formation, but also for maintenance of established tumors (Barakat et al., 2013). Established MB flank tumors depleted of cilia by tamoxifen-induced removal of Kif3a initially underwent growth arrest and then regressed. In vitro, removal of cilia from MB cells caused cell death that could be
prevented by transfection with GLI2. These results suggested that cilia are necessary for Shh target gene activation by Gli proteins in established tumor cells.

Interestingly, out of 24 human MB samples analyzed, primary cilia were identified almost exclusively in tumors of the SHH and WNT subgroups, but not in Group 3 or Group 4 tumors (Han et al., 2009). The presence of primary cilia in SHH tumors is not surprising based on the studies described above. However, the role of primary cilia in Shh-dependent tumors that are resistant to Smo antagonists has not been explored.

**Ofd1 in ciliogenesis and ciliopathies**

Ciliary mutations underlie a group of genetic syndromes collectively referred to as ciliopathies. Examples of ciliopathies are polycystic kidney disease, Bardet-Biedl syndrome, and orofaciodigital syndrome 1 (OFD1). OFD1 is an X-linked dominant disease with lethality in males. Females present with a spectrum of symptoms, including malformations of the face, oral cavity, and digits, and often polycystic kidney disease (Ferrante et al., 2001).

The Ofd1 protein localizes to the centrosome and the basal body at the base of primary cilia, as well as the nucleus (Giorgio et al., 2007; Romio et al., 2004). Singla et al. showed that Ofd1 associates with the distal ends of centriolar microtubules and controls centriole length (Singla et al., 2010). Additionally, they demonstrated that Ofd1 is necessary for centriole distal appendage formation and recruitment of the intraflagellar transport protein Ift88, two processes essential for cilium formation. Disease-associated *Ofd1* mutations differentially affect centriole length and ciliogenesis, explaining the wide variety of phenotypes in patients. Developmental studies in mice
show that Ofd1 inactivation is associated with either loss (Bimonte et al., 2011) or
abnormal activation (D'Angelo et al., 2012) of Shh signaling, depending on the tissue
and development stage.

In sum, studies on cilia and Ofd1 mutants in either oncogenic or developmental
Shh signaling suggest that phenotypes are highly context-dependent. Thus, we set out
to explore the consequence of Ofd1 mutations on Shh signaling and drug resistance in
MB.

**Results**

**Ofd1 mutants reveal that loss of primary cilia can cause resistance to Smo
inhibition**

Since Ofd1 is reported to promote cilia formation, we asked if Ofd1 mutants
identified in the resistance screen display changes in primary cilia. Indeed, all three
Ofd1 mutant clones (GD10, GG6, and LE2) exhibit reduced numbers of primary cilia
compared to parental and Sufu mutants from the screen (Figure 3.1A,B). In
transmission electron microscopy studies, we never detected primary cilia in any of the
Ofd1 mutant cells, although they were clearly evident in parental SMB21 cells (Figure
3.1C). We found that Ofd1 mutants maintained the characteristic structures of
centrioles, which act as basal bodies to assemble primary cilia during interphase.
Figure 3.1 *Ofd1* mutants have significantly reduced numbers of primary cilia. (A) Representative immunostaining of parental and resistant mutant cells. Acetylated tubulin (green) marks the ciliary axoneme and γ-tubulin (red) marks basal bodies at the cilium base. Nuclei are marked by DAPI. Scale bar, 10 μm. (B) Quantification of cilia immunostaining shown in (A), based on percent ciliated DAPI-positive cells. Both *Sufu* and *Ofd1* mutants have reduced cilia numbers, but *Ofd1* mutants have significantly fewer cilia compared to both parental cells and *Sufu* mutants. One-way ANOVA with Tukey correction, *p*<0.0001 (n=4 independent experiments, mean ± SEM). (C) Representative transmission electron microscopy (TEM) images of primary cilia (yellow arrows) and centrioles (blue arrows) in parental and resistant mutant cells. The cross-section of a primary cilium has a 9-doublet microtubule structure surrounded by a vacuole space. The side-view of a primary cilium shows the cilium emerging from the basal body formed from one of the centrioles, through a vacuole to outside the cell. The *Ofd1* mutant cells have centriole structures, but do not have primary cilia associated with these structures. Scale bars, 500 nm.
Figure 3.1 (Continued)

A.

Parental  DG8  DB5  GD10  GG6  LE2

Acetylated Tubulin  γ-Tubulin  DAPI

B.

% Cilia

Parental  DG8  DB5  GD10  GG6  LE2

Sufu Mutants  Ofd1 Mutants

C.

Parental  Parental

GD10  GG6

LE2

63
Next, we asked if resistance in *Ofd1* mutants was specific to a unique *Ofd1* function or if it could be broadly expanded to loss of primary cilia in general. To this end, we genetically targeted *Kif3a* and *Ift88*, both of which are important for ciliogenesis (Haycraft et al., 2007; Marszalek et al., 2000). CRISPR/Cas9-mediated depletion of *Kif3a* (Figure 3.2A,B) and short hairpin RNA (shRNA)-mediated depletion of *Ift88* (Figure 3.2C,D) in SMB21 cells resulted in resistance to LDE225. Resistance with *Kif3a* depletion was also confirmed in two independently established MB cell lines (Supplementary Figure S3.1A,B). Furthermore, targeting *Kif3a* and *Ift88* reduced Gli1 protein levels, but precluded further reduction of Gli1 in response to LDE225 (Figure 3.2A,C). These results are consistent with the depletion of *Ofd1* in SMB21 cells (Figure 2.8). Thus, we propose that cilia loss in general may be a mechanism of resistance to Smo targeted therapy in MB.
Figure 3.2 Reduction of primary cilia genes *Kif3a* and *Ift88* causes resistance to LDE225. (A) CRISPR constructs targeting *Kif3a* reduce Kif3a and Gli1 protein levels. Treatment with 1 μM LDE225 (LDE) for 24 hours does not further reduce Gli1 levels. (B) Depletion of Kif3a results in functional resistance to LDE. Relative survival was assessed at 0, 10, 100, and 1000 nM LDE. (C) shRNAs targeting *Ift88* reduce Ift88 and Gli1 protein levels. (D) Ift88 depletion results in functional resistance to LDE. Statistical analysis by one-way ANOVA with Dunnett’s correction for each treatment condition versus 0 nM LDE, *p<0.05, **p<0.005, ***p<0.0005, ++p<0.0001 (n = 3 independent experiments, mean ± SEM).
Ofd1 loss in medulloblastoma cells maintains addiction to active Shh signaling

Primary cilia are required for canonical Shh signaling. Paradoxically, *Ofd1* mutants from the transposon screen have activation of Shh signaling despite having lost their primary cilia (Figure 2.4). We found that the level of Shh signaling as measured by Gli1 mRNA and protein in the mutants correlated with the growth rate of the mutants, such that higher Gli1 levels were associated with higher growth rates (Figure 3.3A). To determine if *Ofd1* mutant cells are still dependent on Shh signaling downstream of Smo, we pharmacologically blocked the Shh pathway with small molecules reported to function at the level of the Gli transcription factors. Arsenic trioxide (ATO) is proposed to block the functions of Gli2, in part by promoting its degradation. Alternatively, JQ1 is thought to block Gli1 and Gli2 by directly interfering with transcription of these genes at their promoters. Both ATO and JQ1 reduced survival of *Ofd1* mutants from the screen (Figure 3.3B,C). As expected, *Sufu* mutant survival was also reduced, since Sufu functions upstream of the Gli transcription factors.
Figure 3.3 Medulloblastoma cells with Ofd1 mutations exhibit dependence on active Shh signaling. (A) Growth rate of parental and Ofd1 mutant cells correlates with level of Shh activity. Statistics by two-way ANOVA are only shown for the last timepoint, ***p<0.0001 versus all other groups (n=3 independent experiments, mean ± SEM). (B,C) Small molecules ATO and JQ1 reduce survival of Ofd1 and Sufu mutants (n=3-5 independent experiments, mean ± SEM).
Next, we tested if genetically targeting the pathway at the level of Gli transcription factors also reduces cell survival. Gli2 is the main transcriptional effector of Shh signaling and is responsible for activating Gli1. Therefore, we genetically targeted Gli2 in the resistant mutants. shRNA-mediated knockdown of Gli2 in both Sufu and Ofd1 mutant cells reduced cell survival, indicating that the mutant cells remain functionally dependent on Shh signaling (Figure 3.4A,B). Gli2 knockdown also reduced Gli1 mRNA levels, confirming suppression of pathway activity in the context of reduced cell survival (Figure 3.4C). In contrast, Sufu and Ofd1 mutants were less responsive to Smo knockdown, which is expected since they are resistant to pharmacologic Smo inhibition (Supplementary Figure S3.2). Importantly, these experiments show that both parental and mutant cells are still susceptible to pharmacologic and genetic targeting downstream of Smo at the level of the Gli2 transcription factor. Therefore, we reasoned that Gli2 may play a critical role in activation of Shh signaling in the context of primary cilia loss and Smo inhibitor resistance.

Full-length Gli2 protein (Gli2FL) can either be processed into an activator form (Gli2A) or a truncated transcriptional repressor (Gli2R). Hh activation promotes expression and function of Gli2A, while inhibiting formation of Gli2R. Pathway inhibition tips the balance in the opposite direction—reducing Gli2A function and promoting Gli2R formation. As expected, parental cells treated with LDE225 showed reduced Gli2FL protein levels (a proxy for Gli2A since there are no commercially-available antibodies that can differentiate Gli2FL versus Gli2A) (Figure 3.4D). Concurrently, LDE225 increased Gli2R levels. Sufu mutants had reduced levels of both Gli2FL and Gli2R, probably because Sufu is known to stabilize Gli transcription factors and prevent their
Figure 3.4 Gli2 transcription factor drives Shh dependence in Ofd1 mutant medulloblastoma cells. (A) shRNAs targeting Gli2 reduced survival of parental, DG8 (Sufu mutant), and GD10 (Ofd1 mutant) cells, normalized to control infections with shLuc. (B,C) shRNAs targeting Gli2 reduced target mRNA levels (B) as well as Gli1 mRNA levels (C), normalized to GAPDH and an shRNA targeting Luc. Statistical analysis for (A-C) done by two-way ANOVA, with Dunnett’s correction, **p<0.0005; ***p<0.0001 (n=3 independent experiments, mean ± SEM). (D) Gli2 full-length (Gli2FL) levels are reduced and Gli2 repressor (Gli2R) levels are increased after 24 hours of treatment with 1µM LDE225 (LDE) in parental cells. Sufu mutants have reduced overall levels of Gli2. Ofd1 mutants maintain Gli2FL and lose Gli2R.
Figure 3.4 (continued)

A. 

% Survival

Parental  DG8 (Sufu)  GD10 (Odf1)

shLuc  shGli2-2  shGli2-3

***  ***  ***

B. 

Gli2 mRNA

Parental  DG8 (Sufu)  GD10 (Odf1)

***  ***  ***

C. 

Gli1 mRNA

Parental  DG8 (Sufu)  GD10 (Odf1)

***  ***  ***

D. 

Sufu mutants  Odf1 mutants

Parental  DB5  DG8  GD10  GG6

LDE  -  -  -  -  -  -

Full-length  ~185kDa

Gli2

Repressor  ~80kDa

Actin

70
degradation. Interestingly, Ofd1 mutant cells maintained Gli2FL levels irrespective of LDE225 treatment. Furthermore, these mutant cells do not have detectable levels of Gli2R, with or without LDE225. Together, these results suggest that Gli2 is responsible for activating Shh signaling downstream of Smo and driving resistance in the context of primary cilia loss.

Clinical implications of primary cilia loss after treatment with Smo antagonists

Our in vitro MB model shows that loss of primary cilia results in unopposed pathway activity and Smo antagonist resistance. To determine if these observations have clinical relevance, we assessed cilia loss in two different examples of de novo drug resistance.

First, we analyzed cilia loss from in vivo resistant tumors. In a previous publication (Zhao et al., 2015), we described that subcutaneous transplantation of parental SMB21 cells into nude mice resulted in the formation of tumors that were responsive to LDE225 (Figure 3.5A). However, by ~40 days of treatment, resistant tumors developed. We collected the resistant (LDE225-treated) and vehicle-treated tumors at the end of the study and quantified cilia numbers. Importantly, the resistant tumors had significantly fewer primary cilia compared to the sensitive tumors (Figure 3.5B,C). This suggests that treatment with LDE225 selects for loss of primary cilia in resistant tumors and that primary cilia loss may contribute to in vivo resistance.
Figure 3.5 Resistant *in vivo* medulloblastoma and clinical BCC samples exhibit loss or mutations in cilia. (A) SMB21 cells subcutaneously implanted into nude mice and treated with LDE225 (LDE) develop resistance within 40 days of treatment (n= 4 vehicle and 5 LDE treated mice, mean ± SEM). (B) Tumors collected from vehicle and LDE treated animals were immunostained for primary cilia (green = acetylated tubulin, red = γ-tubulin, blue = DAPI). Scale bar = 20 µm. (C) Quantification of cilia immunostaining shown in (B), based on percent ciliated DAPI-positive cells. Averages of 3 images per tumor are plotted as individual datapoints. Resistant tumors have significantly reduced numbers of primary cilia. Midline = mean, whiskers = S.D. T-test, p<0.005. (D) Vismodegib-resistant clinical BCC samples show significantly increased cilia gene mutations compared to untreated samples. Percentages of cilia gene mutations were quantified based on total number of cilia gene mutations normalized to total number of mutations in each sample. Mann-Whitney test, p<0.05 (n= 48 untreated and 11 resistant samples). (E) Recurrently mutated cilia genes are shown as a percent of total number of samples in each population (48 untreated and 11 resistant). Recurrence was quantified based on total number of samples with a given mutated cilia gene normalized to the total number of samples in the population. Each dot represents at least one cilia gene. Recurrences for *SMO*, *PTCH1*, and *OFD1* are labeled. (F) Resistant BCCs had significantly more recurrently mutated cilia genes compared to untreated patients. Mann-Whitney test, p<0.0001 (n= 303 cilia genes).
Figure 3.5 (continued)

A. Tumor Volume (mm³) vs Treatment Day

B. Sensitive vs Resistant Images:
   - Acetylated Tubulin
   - γ-Tubulin
   - DAPI

C. % Cilia

D. % Cilia Gene Mutations

E. Mutated Cilia Genes vs % Untreated Samples

F. Mutated Cilia Genes vs % Samples
Next, we asked whether loss of primary cilia is observed clinically in resistant tumors. Due to the location of MBs, it is difficult to surgically collect tumors after treatment resistance. Additionally, the use of Smo antagonists in MB is relatively recent and the population of patients is relatively small, made even smaller by stratifying patients based on SHH subgroup tumors. In contrast, BCCs are easier to collect, all BCCs are dependent on the Shh pathway, and there are more patients who have been treated with Smo antagonists since the early clinical trials described in Chapter 1. Thus, we asked if cilia mutations are more likely to occur in resistant versus untreated BCCs.

A recent study reported whole-exome sequencing of BCCs that had developed resistance to the Smo antagonist vismodegib (Sharpe et al., 2015). We analyzed this dataset for mutations in cilia genes, using the SYSCILIA gold standard list of 303 genes for known ciliary components (van Dam et al., 2013). We also analyzed a control dataset of untreated BCCs, reported in the same study. We found that the percent cilia gene mutations out of total mutations per sample were significantly higher in the resistant (n=11) versus the untreated (n=48) populations (Figure 3.5D). Our cilia gene list included SMO, since it is known to function in the primary cilium. Recognizing that SMO mutations were more common in the resistant samples (7 out of 11) compared to the untreated (3 out of 48), we reanalyzed the datasets excluding SMO mutations from the analysis and still found that resistant tumors had significantly more cilia gene mutations (Supplementary Figure S3.3A). Finally, we analyzed these datasets for recurrently mutated cilia genes in resistant versus untreated samples (Figure 3.5E). The percent of samples with PTCH1 mutations was high (>60%) and did not change between resistant and untreated samples. This is expected because PTCH1 is the most
common driver in BCC and thus would be present before and after treatment. Also as expected, SMO was recurrently mutated in the resistant samples, as reported by Sharpe et al. Interestingly, OFD1 was mutated with predicted loss-of-function in one resistant patient and also in one untreated patient. Overall, there were significantly more recurrently mutated cilia genes in the resistant versus untreated BCCs, regardless of whether SMO mutation recurrence was included in the analysis (Figure 3.5F and Supplementary Figure S3.3B). Based on these results in human BCC tumors, we concluded that cilia mutations are more likely to occur in resistant tumors.

In conclusion, identification of Ofd1 as a candidate resistance gene in the transposon screen (Chapter 2) led us to discover that loss of primary cilia can cause resistance to Smo inhibitors in Shh-dependent MB. We confirmed that depletion of other cilia components (Kif3a and Ift88) also caused resistance. Importantly, resistance by cilia loss was accompanied by activation and dependence on Shh signaling via the Gli2 transcription factor. These findings may have clinical implications, since resistant MBs exhibit loss of cilia in vivo, and resistant BCCs from patients have increased incidence of cilia mutations.

**Discussion**

**Reactivation of Shh signaling following primary cilia loss is mediated by the Gli2 transcription factor**

Previous work has shown that cilia loss in cultured MB cells results in growth arrest that can be rescued by artificial introduction of Gli2 (Barakat et al., 2013). However, short of exogenous Gli2 introduction, the authors concluded that cilia are
continuously needed for proper Shh target gene activation by Gli proteins. Our work offers an alternative scenario and suggests that endogenous Gli2 activation concomitant with cilia loss is a mechanism of resistance to upstream pathway inhibition. Furthermore, we hypothesize that such Gli2 activation can occur because loss of primary cilia abrogates formation of Gli2R but does not inhibit formation of some Gli2FL/Gli2A that can maintain pathway activity. We are currently testing if cells that become resistant due to genetic targeting of Kif3a and Ift88 also maintain Gli2FL but lose Gli2R. This would support our hypothesis that resistance by cilia loss is accompanied by a shift in the Gli2FL/Gli2R ratio. Since Gli2 localizes in the cytoplasm as well as the nucleus, we are also using subcellular fractionation to verify that the Gli2 present in cilia mutants can localize to the nucleus, where it would be able promote target gene transcription.

Unlike previous reports that stressed loss of Gli3R in the absence of cilia, we do not think Gli3 is playing a major role in SMB cell resistance. We did not detect Gli3 mRNA or protein in the parental or Ofd1 mutant cells. In contrast, we readily observed Gli2 mRNA and Gli2FL protein in the Ofd1 mutants. It is possible that in other contexts where Gli3 plays a more prevalent role, loss of Gli3R would also promote resistance in the context of cilia loss. Alternatively, it is possible that the reason SMB cells developed resistance was because they lacked Gli3 expression and predominantly relied on Gli2A and Gli2R forms for transducing Hh signals.
**Clinical implications for resistance by cilia loss**

Reactivation of Shh signaling has been reported in several studies of Smo inhibitor resistance. Two independent groups recently showed that pathway reactivation primarily driven via SMO mutations is the most common resistance mechanism in human BCCs (Atwood et al., 2015; Sharpe et al., 2015). However, they also reported that intra-tumor heterogeneity could lead to different genetic mutations that converge on reactivating Shh signaling, including multiple SMO mutations, SUFU loss, and/or GLI2 amplification within the same patients. Our work presents cilia loss as another mechanism by which Shh signaling can be reactivated to drive resistance, and we predict that this can co-occur with other genetic drivers of resistance in the same patients. Our human data analysis supports this hypothesis, since we found increased cilia mutations in the same samples of resistant tumors as those that were reported to have resistant SMO variants. Since cilia function at the level of and downstream of Smo, it would be interesting to see if SMO and cilia mutations can co-occur in the same resistant cells and how the level of Shh activity compares to single mutant cells.

As mentioned, OFD1 mutations were identified in two patients. One untreated patient had two missense mutations in the OFD1 gene, one of which was predicted to be deleterious and thus a loss-of-function. Based on our work, we predict that the untreated patient who had a loss-of-function OFD1 mutation would have primary resistance to Smo antagonists. Interestingly, one treatment resistant patient had loss-of-function mutations in both OFD1 and SUFU. This genetic background is similar to the GD10 and GG6 resistant clones from our transposon screen, which had copy loss of Sufu in addition to Ofd1 mutation. Thus, SUFU and OFD1 can both be mutated in the
same patient, just like they were both altered in our resistant cells. We are currently testing if Sufu heterozygosity increases Shh signaling with cilia loss, which could increase the proliferation capacity of resistant tumors (Figure 3.3).

**Conclusion**

In this chapter, we show that cilia loss can cause resistance to Smo antagonists by reactivating Shh signaling downstream of Smo. We also propose a mechanism in which the Gli2 transcription factor mediates Shh signaling upon cilia loss, unopposed by Gli2R. Finally, we provide evidence from *in vivo* studies and human data showing that cilia loss and/or mutations can occur *de novo* after Smo antagonist treatment.

**Methods**

**In vitro cilia staining**

Cultured cells were fixed in 4% PFA, permeabilized with PBS containing 0.2% Triton X-100, blocked with PBS containing 0.1% Triton X-100 and 5% NGS, then incubated with primary and secondary antibody solutions and DAPI. Primary antibodies are specific for Gamma-Tubulin (Sigma Aldrich T5192, 1:250) and Acetylated Tubulin (Invitrogen 322700, 1:250). Secondary antibodies are goat anti-mouse or anti-rabbit conjugated to Alexa Fluor-488 or -568 (Invitrogen, 1:200), or Cy5 (Jackson ImmunoResearch Laboratories, 1:200).
Electron microscopy

0.5-1×10^6 cells were collected, fixed (2.5% paraformaldehyde, 5% glutaraldehyde, 0.06% picric acid in 0.2 M cacodylate buffer), resin embedded, and sectioned (~60 nm). Sections were picked up onto copper grids stained with lead citrate and examined with a TecnaiG^2 Spirit BioTWIN microscope. Images were recorded with an AMT 2k CCD camera.

Cell survival assays with pharmacologic inhibition

Assays were performed as described in Chapter 2 Methods, with the exception that cells treated with ATO or JQ1 were incubated for 96 hours, instead of 72 hours following drug addition. Arsenic Trioxide (ATO) was purchased from Sigma Aldrich. JQ1 was purchased from Cayman Chemicals.

shRNA and CRISPR/Cas9-mediated depletion

Targeted depletion of Kif3a was achieved using the lentiCRISPR vector system (Shalem et al., 2014). shRNA-mediated depletion of Ift88 and Gli2 was achieved using the pLKO lentiviral shRNA system. Infected cells were selected with 1 µg/mL puromycin for 2-4 weeks before downstream experiments. For Gli2 depletion, cells were plated with virus, selected in puromycin 24 hours after plating, and either assayed for survival by MTS assay or collected for RNA extraction after 4 days in virus.

Growth Curve Measurement

2.5 x 10^5 SMB cells were seeded in 6-well plates at day 0. Cells were counted at day 2 and 5 by Trypan Blue exclusion.
Animals

All experimental procedures were done in accordance with the National Institutes of Health guidelines and were approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee. *nu/nu* mice (Charles River Laboratories).

Orthotopic transplantation and *in vivo* treatment

5 x 10^6 cells in 100 µL were injected subcutaneously into the right flank of *nu/nu* mice (6-8 weeks old). Tumor volumes were measured 2 times per week and calculated using the formula \( V = 0.5 \times a \times b^2 \), where \( a \) and \( b \) are the shortest and longest perpendicular tumor diameters, respectively. When tumors reached 150 mm³, animals were randomly separated into treatment groups (5 mice per group). LDE225 was administered at 80 mpk by oral gavage once daily. LDE225 was formulated as diphosphate salt in 0.5% methylcellulose and 0.5% Tween 80 (Fisher). Mice were euthanized when tumors exceeded 2,000 mm³.

In *vivo* cilia staining

Tissues were fixed in 10% formalin, paraffin embedded, and sectioned at a thickness of 5 µm. Paraffin sections were stained with Gamma-Tubulin, Acetylated Tubulin and DAPI.
BCC human data analysis

Publicly available whole-exome sequencing data of resistant and untreated BCC samples were analyzed based on reported single nucleotide variants and insertions/deletions. Original data doi: 10.1016/j.ccell.2015.02.001.

Statistical analysis

Statistical analyses were performed with the Student's t test, one-way ANOVA, or two-way ANOVA with Tukey, Dunnett's, or Mann-Whitney test, as indicated. A p value of 0.05 or less was considered statistically significant. All data analyses were performed using Microsoft Excel or GraphPad Prism 6.
CHAPTER 4: A SMALL MOLECULE SCREEN IDENTIFIES HDAC INHIBITORS AS POTENTIAL THERAPEUTICS FOR TREATING SHH SUBGROUP MEDULLOBLASTOMA

Contributions: Ekaterina Pak wrote chapter, prepared figures, and generated data for all Figures.

Emily Chadwick generated data for Figure 4.5.

Hong Tiv generated data for Figure 4.6.

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**Introduction**

**Targeting Hh signaling beyond Smo inhibition**

As discussed in Chapters 1-3, primary and secondary resistance to Smo inhibitors can occur. Therefore, other targeted therapies have been proposed for treating Hh-dependent tumors. Two FDA-approved drugs have been identified as inhibitors of the Shh pathway: the antifungal agent itraconazole and arsenic trioxide (ATO), which is used for treatment of acute promyelocytic leukemia (Kim et al., 2010b; Kim et al., 2010a). Itraconazole is proposed to inhibit Smo at a site distinct from the critical site bound by cyclopamine, vismodegib, or sonidegib. ATO inhibits Gli2 ciliary accumulation and promotes its degradation. Both single and combination treatment with itraconazole and ATO inhibits Hh signaling and tumor growth mediated by wild-type and vismodegib-resistant Smo (Kim et al., 2013). ATO alone or in combination with itraconazole may also inhibit tumor growth associated with Gli2-mediated resistance. However, for some Smo mutant tumors, these drugs show only partial inhibition or eventual tumor regrowth. Both itraconazole and ATO have entered clinical trials for BCC.

Recently, inhibition of BET bromodomain proteins (BRD2-BRD4 and BRDT) has been proposed for targeting Hh pathway activation in tumors (Long et al., 2014; Tang et al., 2014). BET proteins facilitate gene transcription by binding to specific chromatin sites and interacting with transcription elongation factors and RNA polymerase II. Tang et al. showed that Brd4 occupancy at Gli1 and Gli2 promoters was increased with Hh pathway activation and blocked by the BET protein inhibitor JQ1. Either Brd4 knockdown or addition of JQ1 reduced cell viability, proliferation, and Hh activity in patient-
derived tumor cells that were resistant to Smo inhibitors by mechanisms including SMO or SUFU mutations and amplifications of GLI2. JQ1 also reduced growth of Smo inhibitor responsive and resistant medulloblastoma (MB) and basal cell carcinoma (BCC) tumor allografts, while increasing animal survival. However, ectopic expression of an N-terminally truncated active form of human GLI2 (Gli2ΔN) ablated JQ1-induced inhibition of cell proliferation, suggesting that GLI-responsive promoters that do not require BRD4 for their transactivation exist and are sufficient to overcome BET inhibition. A different study also found that JQ1 was effective at reducing cell proliferation and tumor growth of MYC-amplified MBs that were not of the SHH subgroup (Bandopadhayay et al., 2014). Thus, BET inhibitors may be applicable for targeting several oncogenic pathways in MB tumors.

Several other inhibitors that work downstream of Smo offer alternatives for treating resistant tumors. Small molecules that block Gli function either directly or by blocking Gli interaction with co-activators have been proposed (Bosco-Clement et al., 2014; Lauth et al., 2007). Targeting Hh signaling at the cAMP-dependent PKA node is another strategy. For instance, phosphodiesterase inhibitors can block cAMP degradation and thereby increase activity of the negative Hh pathway regulator PKA (Williams et al., 2015). In vivo phosphodiesterase inhibition was shown to oppose the growth of Smo inhibitor resistant MB (Ge et al., 2015) as well as Shh-dependent MB driven by loss of Gnas (He et al., 2014b). In considering these alternatives, demonstration of target specificity and/or specificity for Hh-dependent tumors will be important.
**HDACs in Hh signaling**

There is growing evidence that HDACs can modulate Hh signaling both during normal development and in tumors. While some studies have looked at specific HDAC isoforms, others have only examined the effects of general HDAC inhibitors and thus could only draw general and drastically simplified conclusions of HDAC effects on the pathway. Still, there are several studies that offer noteworthy examples of HDACs in Hh signaling.

Canettieri et al. proposed that HDACs are involved in a positive feedback loop, whereby Hh activation increases HDAC levels and this promotes HDAC-mediated deactylation and activation of Gli transcription factors (Canettieri et al., 2010). They presented several lines of evidence for this model. First, they showed that treatment of cerebellar granule neuron precursors (GNPs) with Shh increased levels of HDAC1 protein and mRNA, as well as GNP proliferation. HDAC inhibition antagonized proliferation and reduced Gli1 mRNA and protein levels. To support involvement of Gli acetylation in their model, Canettieri et al. showed that a non-acetylatable full-length Gli1(K518R) mutant enhanced Hh transcriptional activity compared to wild-type Gli1, and HDAC1 did not further enhance this activity. They concluded that HDAC-mediated deacetylation of Gli1 confers enhanced transcriptional activity. To turn off the HDAC1 activation loop, Canettieri et al. suggested that a Cul3-REN E3 ubiquitin ligase complex targets HDAC1 for degradation.

This group also showed that HDAC regulation of Hh activity occurs both during development and in disease. HDAC1 and Gli1 were predominantly expressed in the outer external germinal layer (EGL) of the developing cerebellum, where highly
proliferating GNPs reside. Treatment of 4-day-old mice with the HDAC inhibitor TSA antagonized Hh signaling and reduced GNP proliferation. Additionally, knock-down of \textit{HDAC1} by siRNA reduced proliferation of human MB cells \textit{in vitro}. Interestingly, there is evidence for frequent genetic loss of the HDAC1 inhibitor \textit{REN} (Di Marcotullio et al., 2004) and significantly higher levels of \textit{HDAC1} in human MB compared with normal cerebellum.

A more recent study found further support that Shh-dependent MBs have increased HDAC activation (Lee et al., 2013). Aberrant Shh stimulation of GNPs maintained high HDAC activity, which was associated with proliferation \textit{in vitro} and hyperplasia of the EGL \textit{in vivo}. Treatment with HDAC inhibitors blocked GNP proliferation. Interestingly, if GNPs were not stimulated by Shh, they began to differentiate and lose HDAC activity.

HDACs may also regulate Hh signaling via the repressive roles of Gli3 and Sufu. Gli3 repressive function may involve binding by the Ski corepressor and recruitment of HDAC1, possibly leading to histone deacetylation (Dai et al., 2002). Another study found that Sufu can inhibit Gli-mediated transcriptional activation in the nucleus by recruiting an HDAC corepressor complex to Gli target genes (Cheng and Bishop, 2002). In addition to direct interaction and coregulation between HDACs and Hh pathway components, HDACs may be able to regulate other agonists and antagonists of Hh signaling and thus indirectly affect pathway output.

In sum, the roles of HDACs with relation to canonical Hh signaling are diverse and complex. Depending on the context, they can promote or oppose Hh target gene
transcription. Thus, further exploration of HDACs in normal and oncogenic Hh signaling would be highly beneficial.

**Results**

**Primary screen results**

A small molecule screen was conducted on Ptch\(^{+/−}\) mouse-derived MB cells (SMB21 cells) with a collection of 960 compounds containing inhibitors targeting GPCRs, kinases, and epigenetic modifiers. Each compound was tested at two concentrations: 1 µM and 10 µM for GPCR targets or 0.1 µM and 1 µM for kinase and epigenetic targets. The screen was conducted in 3 sessions. Each session included standards plates that generated a linear response between cell number and absorbance by MTS assay (Figure 4.1A). Negative controls DMSO and two MEK inhibitors were included on each screening plate, while the positive control Smo inhibitor LDE225 reduced cell survival by > 50% in all three screening sessions (Figure 4.1B). We identified 111 inhibitors that reduced cell survival by at least 50% at 1 µM based on the mean of 2 replicates (Figure 4.1C,D). An additional 74 inhibitors showed at least 50% reduction at 10 µM.
Figure 4.1 Small molecule screen identifies compounds that reduce medulloblastoma cell survival. (A) Standards plates from each screening session produced a linear response between cell number and absorbance at 490 nm by MTS assay. (B) Positive control inhibitor LDE225 reduced cell survival in all 3 screening sessions by >50%, while negative control MEK inhibitors (CI-1040 and PD325901) maintained cell survival at >90%. Each value represents the mean of experimental replicates ± SD (n varies by control condition and number of plates per screening session). (C,D) Hits were defined as inhibitors that reduced cell survival by >50%, as indicated by the dotted line (n= 2 technical replicates, mean ± SD). DMSO and LDE225 values from each corresponding screening plate were averaged and are highlighted (DMSO = yellow; LDE225 100 nM = green; LDE225 1 µM = red).
Figure 4.1 (continued)

A. Absorbance (490nm) vs. Cells/Well

B. % Survival

C. % Survival

D. % Survival

Legend:
- DMSO
- CI-1040 (0.1uM)
- CI-1040 (1uM)
- LDE225 (0.1uM)
- LDE225 (1uM)
- PD325901 (1uM)

Session 1

Session 2

Session 3

Legend:
- 0.1µM
- 1µM

Legend:
- 1µM
- 10µM
**Secondary screen results**

To confirm primary screen hits and identify inhibitors with potency in the micromolar or submicromolar range, we selected 78 compounds for a 5-dose secondary screen. We removed compounds with non-specific toxicity based on previous screens in the Rubin Lab. We also removed compounds targeting topoisomerases and polymerases and compounds targeting known Shh pathway interactions (e.g. Akt, PI3K, and mTOR). Compounds targeting GPCRs were screened at a dose range of 0.12-10 µM, while those targeting kinases/epigenetic modulators were screened between 0.0625-1 µM. We screened both the parental SMB21 cell line as well as SMB21 cells over-expressing the truncated, constitutively active form of human GLI2 (Gli2ΔN cells). The Gli2ΔN cells are resistant to Smo inhibitors due to downstream activation of the Hh pathway (Zhao et al., 2015).

Standards and control conditions in the secondary screen were consistent with results from the primary screen (Figure 4.1A, B and data not shown). Hits from the dose screen were ranked based on the half-maximal inhibitory concentration (IC$_{50}$) (Figure 4.2A,C). Some compounds reduced cell survival by 70-80% even at the lowest dose, and are listed as highly potent inhibitors for which IC$_{50}$ was not calculated. Compounds that failed to reduce cell survival by at least 80% were excluded from IC$_{50}$ calculations (Supplementary Figure S4.1), since we were most interested in pursuing hits with the highest potency.
Figure 4.2 Secondary screen identifies compounds with potent dose-dependent inhibition in SMB21 and Gli2ΔN cells. (A,C) Compounds targeting kinases and epigenetic modulators (A) and GPCRs (C) elicit a range of responses from SMB21 and Gli2ΔN cells based on IC₅₀. Drugs that failed to reduce cell survival by >80% at the highest dose are not shown. Insets list drugs for which IC₅₀ was not calculated due to potent drug response at the lowest dose. HDAC inhibitors, comprising a class of hits from the screen, are highlighted in red. (B,D) Selected drugs show representative dose responses from the secondary screen (n= 2 technical replicates, mean ± SD).
The downstream Hh signaling inhibitor JK 184, as well as the Smo inhibitor LDE225, were identified as hits in the dose screen. These hits help validate the secondary screen approach for identifying inhibitors of Shh-dependent MB cells. Additionally, the BET bromodomain inhibitor JQ1, previously reported to target SHH subgroup MB (Tang et al., 2014), was also a hit on the SMB21 cells, with less inhibition seen in the Gli2ΔN cells. Inclusion of Gli2ΔN cells in the secondary screen identified some inhibitors, like JQ1, that could be targeting tumor cell signaling between Ptch1 and Gli2 function. As expected, the Smo inhibitor LDE225 exhibits differential responses in SMB21 versus Gli2ΔN cells (Figure 4.2B). Interestingly, the carrier-mediated anandamide transport inhibitor AM 404, also showed a drastic difference between SMB21 and Gli2ΔN cell responses (Figure 4.2D).

Several HDAC inhibitors were clear hits in the dose screen. Since these comprised a class of inhibitors that worked with high potency on both SMB21 and Gli2ΔN cells, we focused our attention on the use of HDAC inhibitors for the treatment of SHH-MB.

**Select HDAC Inhibitors reduce viability in Shh-dependent medulloblastoma cells**

MB cells did not show global response to all HDAC inhibitors in the screen (Supplementary Table S4.1). The epigenetics collection in the primary screen included a total of 76 compounds targeting HDACs, with a range of specificities for different HDAC classes. We found 9 HDAC inhibitors that were considered hits in the primary screen because they reduced viability by >50% in SMB21 cells at 1 µM (Figure 4.3A). Seven of these belonged to the hydroxamic acid chemical class (SB939, JNJ-
26481585, LBH-589, Belinostat, TSA, CUDC-101, and PCI-24781), while Trapoxin A and HC Toxin belonged to the cyclic tetrapeptide class. Four of these (JNJ-26481585, LBH-589, PCI-24781, and SB939) have a similar chemical structure not shared by the other 76 inhibitors tested. The aliphatic acid and benzamide classes of inhibitors did not produce hits.

We excluded HC Toxin for possible off-target toxicity and tested the remaining 8 HDAC inhibitors in the dose-screen. All 8 HDAC inhibitors showed a maximum response at or below 1 µM (Figure 4.3B). Interestingly, we found some activity with HDAC inhibitors against Gli2ΔN cells. Currently, there are no targeted therapies available for SHH-MB with reactivation of the pathway downstream of Smo. Thus, a therapeutic that could target GLI2-amplified tumors as well as tumors with loss of SUFU or resistant SMO mutations is highly desirable.
Figure 4.3 Select HDAC inhibitors show dose-dependent inhibition in SMB21 and Gli2ΔN cells. (A) HDAC inhibitors with the cyclic tetrapeptide and hydroxamic acid chemical structures reduced survival of SMB21 cells by >50% (dotted line) at 1 μM in the primary screen. Four replicates of JNJ-26481585 and 2 replicates of all other compounds were screened. (B) All 8 of the HDAC inhibitors re-tested in the secondary screen show reduction in survival of both SMB21 and Gli2ΔN cells (n= 2 technical replicates, mean ± SD).
Figure 4.3 (continued)

A. Concentration = 0.1μM  
Concentration = 1μM

B. 
- Trapoxin A
- CUDC-101
- Trichostatin A
- LBH-589
- PCI-24781
- Belinostat
- SB-939
- JNJ-26481585

% Survival vs. Concentration (nM)
We chose two HDAC inhibitors to test for time-dependent effects on viability at 24, 48 and 72 hours in SMB21 cells (Figure 4.4A,B). We also tested a human SHH subgroup MB cell line (M311) that is unresponsive to Smo inhibition. Both cell lines responded to JNJ-26481585 (JNJ) and LBH-589 (LBH) with IC₅₀’s in the nanomolar range. In contrast, SAHA, an inhibitor that was not a hit in the screen, required higher doses to achieve reduced viability at these timepoints and had IC₅₀’s that were an order of magnitude greater than for JNJ and LBH (Figure 4.4C).
Figure 4.4 HDAC inhibitors induce nanomolar range, time-dependent survival reduction in mouse and human medulloblastoma cells. (A,B) Mouse-derived SMB21 and human M311 cells treated with JNJ-26481585 (A) or LBH-589 (B) begin to show a dose-dependent response at 24 hours and reach maximal response by 48 hours with inhibitor. (C) SAHA requires higher doses to exert inhibitory responses in SMB21 and M311 cells (n= 3 independent experiments, mean ± SEM).
Figure 4.4 (continued)

A.  

JNJ-26481585  

<table>
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<tr>
<th>Time (h)</th>
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</table>

SMB21  

% Survival  

Concentration (log[nM])

M311  

% Survival  

Concentration (log[nM])

B.  

LBH-589  

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<thead>
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SMB21  

% Survival  

Concentration (log[nM])

M311  

% Survival  

Concentration (log[nM])

C.  

SAHA  

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<thead>
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<td>598.4</td>
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SMB21  

% Survival  

Concentration (log[nM])

M311  

% Survival  

Concentration (log[nM])
The HDAC inhibitor JNJ-26481585 shows specificity in tumor cells

As described in Chapters 2 and 3, Shh-dependent MB can develop resistance to Smo inhibitors. To assess whether JNJ could be used to treat tumors that become resistant by mechanism other than GLI2 amplification, we tested SMB21 cells that acquired resistance either through transposon mutagenesis or by over-expression of constitutively active human HRAS(G12V) (Zhao et al., 2015). While both the Shh-dependent DG8 (transposon insertion in Sufu) and GD10 (transposon insertion in Ofd1) cells respond to JNJ similarly to parental SMB21 cells (IC$_{50}$ = 8.2 nM, 5.482 nM, and 7.601 nM, respectively), the HRAS(G12V) cells, which do not depend on active Shh signaling, had an increased IC$_{50}$ (31.23nM) (Figure 4.5A). Thus, there is some specificity for JNJ depending on the signaling pathway driving tumor cell survival in MB cells.

To further examine JNJ specificity and explore the potential of HDAC inhibitors for treating brain malignancies, we assessed the effects of JNJ on normal mouse brain. Since the brain is considered a predominantly post-mitotic organ (although there are regions with neural stem cells that are capable of proliferation and differentiation), we were most interested in asking if HDAC inhibitors induce significant cell death. We sectioned and treated wild-type mouse sagittal organotypic slice cultures with DMSO, LDE225 (LDE), or JNJ for 7 days and measured cell death by TUNEL. As mentioned in Chapter 1, LDE has been used successfully in the clinic to treat MB with an acceptable toxicity profile and thus serves as a good negative control for apoptotic drug effects on the brain. Treatment with JNJ well above the IC$_{50}$ for SMB21 and M311 cells (see Figure 4.4A) did not significantly induce cell death in the cerebellum, the brain region
where SHH subgroup MBs develop (Figure 4.5B,C). However, treatment with a very high dose of JNJ induced significant cell death. Thus, there may be a therapeutic window in which HDAC inhibitors like JNJ can have anti-tumor effects without causing significant damage to the surrounding normal brain tissue. Interestingly, none of the drug conditions significantly increased cell death compared to DMSO in the cortex (Supplementary Figure S4.2). This suggests that different brain regions may respond differently to HDAC inhibitors and that careful monitoring of region-specific side effects will be important.
Figure 4.5 JNJ-26481585 displays potential for therapeutic specificity in tumor cells. (A) Smo inhibitor resistant DB5 (Sufu mutant) and GD10 (Ofd1 mutant) cells respond to JNJ-26481585 (JNJ) with a similar inhibition profile to parental SMB21 cells. However, Ras-dependent cells require a higher concentration of JNJ for inhibition. Two-way ANOVA with Tukey correction, ***p<0.0001 for HRAS(G12V) versus all other groups, with the exception of Ofd1 mutant cells at 50 nM, where ++p=0.0005 (n=3 independent experiments, mean ± SEM). (B) Quantification of TUNEL positive cells shown in (C) indicates that JNJ treatment at 20 nM and 50 nM does not significantly induce apoptosis in normal brain compared to DMSO and LDE conditions, while 500 nM JNJ induces significant apoptosis. One-way ANOVA with Tukey correction, ***p<0.0001 (n=4 independent experiments with 3-4 slices per treatment group and 3 images per slice, mean ± SEM). (C) P6 wild-type brain slice cultures were treated with the indicated conditions for 6 days and stained for apoptosis with TUNEL (red) and for nuclei with DAPI (gray). Representative 60X images of the cerebella are shown. Scale bar, 50 µm.
Figure 4.5 (continued)

A.  

<table>
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<th>Cell Line</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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<td>SM21</td>
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</tr>
<tr>
<td>SFU Mutant</td>
<td>8.2</td>
</tr>
<tr>
<td>OFD1 Mutant</td>
<td>5.482</td>
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<tr>
<td>HRAS(G12V)</td>
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</tr>
</tbody>
</table>

(B)  

% Survival vs log [JNJ-26481585]

(C)  

DMSO

1µM LDE

20nM JNJ

500nM JNJ
The HDAC inhibitor JNJ-26481585 shows efficacy in vivo

Having shown specificity and efficacy in vitro, we next asked if JNJ works to reduce tumor burden in vivo. First, we assessed tolerability for JNJ alone and in combination with LDE. Mice treated with 10 mg/kg of JNJ for 2 weeks had a 2.3% loss in body weight on average (Figure 4.6A). To determine the optimal dose for combination treatment, we administered a dose escalation of JNJ (5-10 mg/kg) with 60 mg/kg LDE. During the course of combination treatment with 10 mg/kg JNJ, 3 out of 5 mice lost 10% or more of their initial body weight and exhibited lethargic behavior. Thus, we chose a dose of 8 mg/kg for JNJ in the efficacy study. Due to weight loss concerns in the following efficacy study, we further reduced the combination dose of JNJ to 4 mg/kg after day 21, but not in the single-treatment group (Figure 4.6B).
Figure 4.6 JNJ-26481585 inhibits tumor growth and improves survival in a xenograft model of medulloblastoma. (A) JNJ alone maintained body weight at above 12% over a 14 day treatment period (n=3 mice). A dose-escalation of JNJ (5 mg/kg at day 0, 8 mg/kg at day 15 (first arrow) and 10 mg/kg at day 21 (second arrow) suggested that the best tolerated combination dose was with 8 mg/kg JNJ (n=5 mice). (B) All drug treatments (LDE at 60 mg/kg and JNJ at 8 mg/kg) reduced body weight in mice (n=10 per group). The arrow represents day 21, the last day at which combination-treated mice were treated with 8 mg/kg JNJ; from day 22 until the end of the study, these mice were treated with 4 mg/kg JNJ. (C) All drug conditions blocked SMB21 xenograft tumor growth compared to vehicle. (D) Kaplan-Meier survival analysis indicates that all drugs extended survival of animals compared to vehicle.
Figure 4.6 (continued)

A. % Body Weight

B. % Survival

C. % Tumor Volume

D. % Survival
Tumors grew for all vehicle-treated mice, and 7 out of 10 had to be sacrificed due to tumor burden before the end of the 50-day treatment (Figure 4.6C,D). Single treatment with 60 mg/kg LDE reduced tumor growth and maintained survival of all animals to the end of the 50 day study. Tumor growth was also reduced for all JNJ single-treatment animals. Even for the tumors that grew, final volumes were below 2000mm$^3$ and animals were able to survive to the completion of the 50-day endpoint of the study. Thus, single agent JNJ inhibited tumor growth for all animals compared to the vehicle-treated group. Tumor growth was reduced for 9 out of 10 combination-treated animals. One combination-treated animal had a complete response, with no measurable tumor from day 14 to day 50 of treatment. Another combination-treated animal initially showed robust response, but eventually developed resistance starting around day 32 of treatment. The animal that became resistant to treatment had more drug holidays (12 days) than any other animal in the study (average holiday length was 4 days) (see methods). It is possible that the drug holidays and/or reduction in JNJ dosage contributed to the resistance observed in this animal.

**HDAC inhibition can block Shh signaling and may be important for tumor response to Smo inhibitors**

Since we observed some specificity of HDAC inhibitors targeting Shh-dependent MB cells, we investigated the mechanism by which these compounds could block oncogenic pathway activity. We tested 5 of the 8 HDAC inhibitors that were hits in the dose screen and found that all were capable of reducing Gli1 in both the SMB21 cells and the Smo inhibitor-resistant Gli2ΔN cells (Figure 4.7A-C). Since these inhibitors can
target multiple HDACs, we next wanted to determine if specific HDACs could target Shh signaling. Previous reports indicate that HDAC1 and HDAC2 promote Hh-induced transcriptional activation (Canettieri et al., 2010). Thus, we started by looking at HDAC1 and HDAC2 regulation with respect to Shh signaling in our SMB21 cells. Preliminary results indicate that knockdown of HDAC1 inhibits the proliferation of SMB21 cells (Figure 4.7D). The reduced proliferation is accompanied by a reduction in Gli1 protein levels (Figure 4.7E). Notably, knockdown of Smo may reduce HDAC1 and HDAC2 levels, as well (Figure 4.7E and Supplementary Figure S4.3).
Figure 4.7 HDACs promote oncogenic Shh signaling. (A-C) HDAC inhibitors reduce levels of the Hh target protein Gli1 in both SMB21 and Gli2ΔN MB cells after 24 hours of treatment with 1 µM inhibitor. (D) Knockdown of HDAC1 inhibits proliferation of SMB21 cells in vitro. Uninfected cells grown in puromycin (PURO) and Smo knockdown serve as positive controls (mean of 3 replicates from one experiment). (E) Cell lysates collected in parallel with the last day of growth measurement in (D) confirm HDAC1 knockdown and show reduction in Gli1 protein. Smo knockdown also reduces Gli1 and HDAC1 protein levels. (F) Combination treatment with LDE225 (LDE) and JNJ-26481585 (JNJ) does not consistently exhibit synergistic effects on survival. Two-way ANOVA, *p<0.05; **p<0.005; ***p<0.0005 for single treatments compared to DMSO and ++p<0.05 for synergistic effect (n=3 independent experiments, mean ± SEM). (G) Boxplot representation comparing reads per kilobase of transcript per million mapped reads (RPKM) for HDAC2 in resistant BCCs, sensitive BCCs, and normal skin. Kruskal-Wallis with Dunn’s correction, *p<0.05 compared to normal skin. The box represents the first and third quartiles, with whiskers representing the range (Tukey boxplot). Center line, median. Data obtained from Atwood et al., 2015.
Since inhibition of either HDACs or Smo impinges on Shh signaling and proliferation of SMB21 cells, we asked if combining an HDAC inhibitor with a Smo inhibitor would result in a synergistic reduction in tumor cell survival. Overall, we found only a slight synergistic effect from combination of JNJ and LDE, with a p-value slightly above the threshold of significance (p=0.05007), driven by two of the six combinations tested (Figure 4.7F). These results suggest that HDAC inhibitors may be functioning on the same signaling axis as Smo inhibitors. It is even possible that downstream HDAC inhibition is important for the anti-tumor effects of Smo inhibitors that are currently in clinical use.

Further evidence that HDACs may be on the same pathway downstream of Smo comes from clinical studies in BCC. Interestingly, we found that resistant human BCCs had high HDAC2 mRNA levels despite ongoing treatment with vismodegib and that this up-regulation was significant compared to matched normal skin samples (Figure 4.7G). The pattern of HDAC2 expression mirrored that seen with GLI1 mRNA from the same dataset, in that sensitive tumors had slightly elevated levels of GLI1 while resistant tumors had significantly elevated GLI1 compared with normal skin (Atwood et al., 2015). We are actively investigating whether over-expression of HDAC2 in SMB21 cells will suppress or abolish the anti-tumor effects of Smo inhibition. In this way, we may find a novel mechanism of resistance with supporting clinical evidence.
Discussion

Strategy and results of small molecule screen for medulloblastoma treatment

As discussed in Chapter 2, resistance to Smo inhibitors in Shh-dependent tumors will be a problem for some patients. With this in mind, we chose to focus on identifying new lines of therapeutics that could be used for resistant cases. To this end, we included Gli2ΔN cells in the secondary screen and identified small molecules that had dose-dependent effects even on cells that no longer rely on upstream Shh signaling for pathway activity (Supplementary Figure S4.1). In follow-up studies we also tested other resistant cell lines, including human SHH-MB cells and cells that became resistant by transposon mutagenesis.

Hits from the screen corroborated and built on emerging treatment approaches for MB in preclinical development. A recent study from Tang et al. reported the use of BET bromodomain inhibitors, and specifically JQ1, for targeted inhibition of Shh pathway activation in tumors. Over-expression of a Myc-tagged N-terminally truncated constitutively active form of human GLI2 under the CMV promoter (Gli2ΔN) in mouse-derived Ptch1+/−, p53−/− MB cells opposed growth inhibition by JQ1. The Gli2ΔN cells in our dose screen also express an N-terminally truncated human GLI2 under the CMV promoter. We confirmed that JQ1 reduced cell survival of SMB21 cells more readily than the Gli2ΔN cells (Figure 4.2 and Supplementary Figure S4.1). In contrast, we found that Gli2ΔN cells respond to at least 7 of the 8 HDAC inhibitors re-tested from the primary screen (Figure 4.3). Tang et al. propose that the ectopic Gli2ΔN rescue indicates that proliferative inhibition by JQ1 is mediated largely through Gli transcription and that Brd4-independent transcriptional targets of Gli can overcome BET inhibition.
By this line of reasoning, it is possible that HDAC inhibitors block Gli2 activity instead of transcription of Gli and thus over-expressing Gli from a plasmid would not rescue HDAC inhibitor effects.

In addition to confirming the reported results of BET inhibition studies, our screen built on previous work suggesting the use of HDAC inhibitors in MB treatment. We vastly expanded the number of HDACs tested for anti-tumor effects and found a set of compounds that worked across several MB cell lines. We found that the hydroxamic acid and cyclic tetrapeptide classes of HDAC inhibitors, but not benzamides or aliphatic acids, could reduce survival of MB cells in the primary screen. Hydroxamic acids and cyclic tetrapeptides generally target several classes of HDACs. Benzamides are generally restricted to inhibiting class I HDACs. Thus, our screen has indicated that different structural properties of HDAC inhibitors will be important to understand and harness for targeted treatment in oncology.

Finally, other classes of drugs that came up in the screen included cholesterol synthesis inhibitors and dopamine signaling inhibitors. It is tempting to speculate that the cholesterol synthesis inhibitors may be functioning directly on Smo, since cellular sterol levels can mediate Smo signal transduction (Cooper et al., 2003). However, Gli2ΔN cells with pathway activation downstream of Smo also respond to cholesterol synthesis inhibitors, suggesting more complex mechanisms at play.

**Roles of HDAC1 and HDAC2 in Shh signaling**

Our preliminary data suggests that there may be reciprocal regulation between HDACs and Shh pathway activation, functioning within the same axis. We found that all
5 of the HDAC inhibitors tested reduced Gli1 protein levels in both sensitive and Smo inhibitor resistant (Gli2ΔN) cells. Thus, HDAC inhibition can reduce pathway target genes downstream of Gli2 function. Moreover, knock-down of the upstream pathway component Smo reduced HDAC1 and HDAC2 levels, indicating upstream pathway regulation of these proteins. We did not find synergy between Smo inhibitors and HDAC inhibitors, also suggesting effects functioning on the same axis.

Based on these early studies, we predict a model in which Shh activation regulates HDAC1 and HDAC2 levels in a Smo-dependent manner, and that HDAC1 and HDAC2 in turn regulate transcriptional output as measured by Gli1 levels. There may be several mechanisms by which HDAC1 and HDAC2 can directly promote Hh transcriptional output. First, HDACs may be functioning at the chromatin to promote Hh target gene expression and HDAC inhibition could block these effects. Alternatively, HDAC inhibitors may work directly on Hh signaling components. Interestingly, Canettieri et al. found that SAG stimulation induced recruitment of HDAC1 and HDAC2 to the Ptch1 promoter but also induced hyperacetylation of H3 and H4 in this region of the chromatin (Canettieri et al., 2010). This suggests that HDAC1 and HDAC2 at active Hh target gene promoters are not deacetylating histones. Instead, Canettieri et al. presented evidence that HDAC1 can deacetylate Gli1 at Lys 518, leading to enhanced transcriptional activity. In a follow-up study, this group also showed that the conserved Lys on Gli2 also regulates its function, preventing Gli2 entry into chromatin when acetylated (Coni et al., 2013).
Potential for HDACs as targets in Hh-dependent tumors

HDACs are druggable targets, with several inhibitors already approved by the FDA (West and Johnstone, 2014). There are three classical HDAC classes (I, II, and IV) containing 11 HDACs, with most current inhibitors targeting several HDACs both within and across the classes. However, isoform-specific compounds with proposed selectivity for HDAC3, HDAC6 and HDAC8 have been developed. In considering the use of HDAC inhibitors for any sort of therapy, there are several key concepts to keep in mind (Falkenberg and Johnstone, 2014). First, the reach of HDACs goes beyond histones, having potential to affect post-translational modifications of the over 1,750 proteins that can be acetylated at lysine residues in human cells. Second, histone hyperacetylation does not necessarily translate into elevated transcriptional activation. The traditional view has been that histone acetyl transferases (HATs) activate and HDACs repress transcription by adding or removing acetyl groups to histones and transcription factors, respectively. However, HDAC activity can enhance transcription, depending on the context. Indeed, regulation of gene transcription is subject to a complex interplay of different epigenetic modifications. Third, HDACs function as catalytic subunits of large protein complexes, and this also contributes to another layer of regulation at the level of chromatin and beyond. Finally, in certain contexts, inhibiting HDACs including HDAC1 and HDAC2 can be tumor-promoting. Thus, methods to improve the specificity of treatment, including targeted delivery or new chemicals that target specific HDACs and their complexes, could be beneficial.

If targeted delivery to the tumor is possible, there is still the question of how HDAC inhibitors would affect the rest of the brain in patients with MB. To examine the
effects of HDAC inhibitors on the brain, we adopted a brain slice culturing technique that would allow us to gauge drug effects in different brain regions at once. Our slice culture studies indicate that JNJ does not significantly induce cell death in the cerebellum or cortex at low doses but can cause cell death at the high dose of 500 nM. Based on our MTS results with JNJ across several cell lines (SMB21, M311, Gli2ΔN, DG8, GD10, and HRAS(G12V)), such a high dose would not be required to induce anti-tumor effects. Thus, our data suggests there may be a therapeutic window where HDAC inhibitors like JNJ can be used to treat brain tumors without damaging the normal brain tissue. However, further investigation of effects on other tissues as well as effects on proliferative regions in the adult brain would need to be done.

Notably, HDAC inhibitors have been used in neurology for many years, and the broad-spectrum HDAC inhibitor valproic acid (VPA) is widely used as an anticonvulsant and mood-stabilizer for epilepsy and bipolar disorder, as well as other neurological conditions. Thus, there is precedent in the use of HDAC inhibitors for central nervous system pathologies and the possibility of targeting HDAC functions and/or interactions in MB warrants further exploration.

Conclusion

In this study, we conducted a small molecule drug screen for the treatment of Shh-dependent MB. We identified several compounds previously reported to target Shh-dependent tumors, validating our methodology. We also identified HDAC inhibitors as a class of drugs that has potential in the treatment of both sensitive and resistant
tumors. Finally, we provide evidence that specific HDACs may play a role in the aberrant activation of Shh signaling in tumors.

**Methods**

**Cell survival assays with pharmacologic inhibition**

Assays were performed as described in Chapter 2 Methods, with the exception that cells were seeded at a lower density (1x10^4 cells per well) and drugs were added 24 hours after cell seeding.

**High-throughput small molecule screening**

SMB21 or Gli2ΔN cells (1x10^4) were added to 96-well plates by automated cell seeding. A separate plate of standards with a dilution series of cells was also prepared. 24 hours after seeding, 50 µl of media containing either DMSO or a small molecule were added to the screening plates. Control compounds were included on every screening plate along with 80 test compounds. All concentrations were tested in duplicate. After 72 hours with inhibitor, cell viability was measured using CellTiter 96 Aqueous One Solution (Promega), and calculated as a percentage of control (DMSO-treated cells).

The primary screen was conducted in 3 sessions. Chemicals for the screen were derived from either the NIH Clinical Collection (http://www.nihclinicalcollection.com) or from custom collections assembled at the Harvard Stem Cell Institute. Positive hits were defined as compounds exhibiting ≥ 50% reduction in cell viability. Each screening plate contained 8 replicates of DMSO, 2 replicates of 1 µM control drug, and 1 well with 100 nM control drug. A separate standards plate was included in each screening session.
To calculate cell survival, the MTS signal was converted to cell number based on the standard curve plate and then normalized to the average of the 8 converted DMSO values on the same plate. Reported values are the average of experimental replicates, represented as percent survival. Values that fell below 0% indicate reduction in cell number below background levels of assay signal, as calculated from the standard curve. Therefore, these negative values were re-assigned 0% survival and the corresponding inhibitors were considered primary screen hits because they reduced cell number below 50% of untreated cells.

**Slice culture**

Brain slices from wild-type P6 mice were prepared as previously described (Chadwick et al., 2015). Briefly, whole brains were dissected and embedded into 3% low melting point agarose. Sagittal slices of 200 µm thickness were prepared using a vibratome and placed onto slice culture inserts (Milipore, Cat# PICM0RG50) pre-coated with laminin. Inserts with slices were placed into individual wells of a 6-well plate containing 1.2 mL of slice culture media (2% B-27, 1% N2 supplement, 1% penicillin/streptomycin, 1% Glutamax, and 1.5 mg/mL Glucose in Neurobasal-A Medium minus phenol red) and incubated at 37°C overnight. Slices were transferred into new 6-well plates with appropriate DMSO or drug conditions in slice culture media for 6 consecutive days. On day 7, slices were fixed with 4% PFA, blocked and permeabilized (5% digitonin in 3% BSA) and stained for DAPI (1:1000) and TUNEL using the CF 640R TUNEL Assay (Biotium Cat# 30074). Slices were mounted onto microscope slides with coverslips. Three 60X and one 10X representative images of the cerebellum and cortex
were taken for each slice with the Nikon Eclipse Ni C2si upright confocal microscope. Quantification of 60X images was done with NIS Elements AR 4.20 software for TUNEL-positive and DAPI-positive cells.

**Cell proliferation assay**

1x10⁶ SMB21 cells were infected with lentivirus. 48 hours after infection, cells were selected in 1 µg/mL puromycin. After 48 hours of selection, cells were counted and added to 96-well plates at 1x10⁴ cells/well with puromycin. Remaining cells were added to 12-well plates for parallel collection of cell lysates. Day 0, 2, 4, and 6 MTS readings were taken starting 24 hours after plating. Fold change was calculated by normalizing all MTS signal to Day 1 signal.

**In vivo efficacy and tolerability studies**

For tolerability studies, nu/nu mice were treated once daily with single dose of 10 mpk JNJ-26481585 (Selleckchem Catalog # S1096) intraperitoneally (i.p.) or with combination dose of 60 mpk LDE225 by oral gavage (ChemieTek Catalog # CT-LDE225) with escalating dose of JNJ-26481585. LDE225 was formulated as diphosphate salt in 0.5% methylcellulose and 0.5% Tween 80 (Fisher). JNJ-26481585 was formulated at 1 mg/mL in 10% hydroxy-propyl-β-cyclodextrin, 25 mg/mL mannitol, in sterile water (pH 8.7).

For efficacy studies, ~2x10⁶ cells in 100 µL media and 30% matrigel were injected subcutaneously into the right flank of nu/nu mice (6-8 weeks old). Tumor volumes were measured 2 times per week and calculated using the formula V=0.5 x a x
b^2, where a and b are the shortest and longest perpendicular tumor diameters, respectively. When tumors reached 150 mm^3, animals were randomly separated into 4 treatment groups (10 mice per group): Vehicle, LDE225, JNJ-26481585 (8 mpk), or combination of LDE225 and JNJ-26481585. Any mouse that dropped more than 15% of initial body weight received a drug holiday (skipped treatment day) until weight recovered to below 15% loss. IP fluid was administered to keep animals hydrated. At day 22 of treatment, all combination-treated mice began to receive a lower dose of 4 mg/kg JNJ in order to reduce the necessity for drug holidays in this group. Mice were euthanized when tumors exceeded 2,000 mm^3.

**Statistical analysis**

Statistical analyses were performed with one-way ANOVA or two-way ANOVA with Tukey test and Kruskal-Wallis with Dunn’s test. A p value of 0.05 or less was considered statistically significant. All data analyses were performed using Microsoft Excel or GraphPad Prism 6.
CHAPTER 5: CONCLUSION
Pathway addiction as a unifying theme in Shh-dependent tumors

As described in Chapter 2, the most common mechanism of resistance to Smo antagonists reported in clinical basal cell carcinomas (BCCs) is reactivation of Shh signaling primarily by mutations in the drug target SMO and in some cases by reactivation of signaling downstream of SMO. In some ways, this is not surprising, since all cases of Gorlin syndrome BCCs and sporadic BCCs are driven by over-activation of Shh signaling. In contrast, not all medulloblastomas (MBs) are driven by Shh signaling. Furthermore, preclinical studies in MB models have implicated PI3K/AKT signaling in conjunction with Shh signaling in driving tumor growth and resistance. There is also evidence that Shh signaling can be repressed while RAS/MAPK signaling can take the role of driving tumor growth.

Given the relatively recent use of Smo antagonists in clinical practice and the relatively low incidence of MB, there is still limited comprehensive knowledge of how treatment resistance can emerge. We predicted that yet unreported mechanisms of resistance can occur and applied a genome-wide approach to explore this possibility. Importantly, we report on a novel mechanism by which Shh signaling can be reactivated downstream of Smo by loss of primary cilia and concomitant loss of Gli2R. Interestingly, we also found primary cilia mutations in resistant human BCCs, and thus propose loss of primary cilia as a previously unappreciated and unreported contribution to resistance.

Our work contributes to the growing body of evidence that Shh pathway reactivation is a common theme of resistance in Shh-dependent tumors. Notably, this theme may have reach beyond resistance to other forms of tumor evolution. Namely, pathway addiction may be a characteristic of recurrent tumors following the traditional
treatment approach of surgery, radiation and chemotherapy. Studies on recurrent MB show that tumors do not change subgroup affiliation at recurrence (Morrissy et al., 2016; Ramaswamy et al., 2013). The stability of MB subgroup affiliation applies to all four of the subgroups (WNT, SHH, Group 3, and Group 4) and may indicate that the different subgroups arise from distinct cells of origin. As previously described, in murine models of SHH-MB, the cells of origin are the granule neuron precursors (GNPs) of the external granule layer of the developing cerebellum. The cells of origin for WNT and Group 3 murine models may be in the lower rhombic lip and postnatal cerebellar progenitor cells, respectively. Thus, subgroup identity may be established in the cell of origin and remain stable as the tumors evolve, even during treatment. Interestingly, subgroup stability does not necessarily translate to genomic stability, and in fact tumors may remain affiliated to the same subgroup while switching ‘trunk’ driver mutations. A recent example of this phenomenon was reported in a MB patient who had a PTCH1 driver mutation that was clonally dominant in the primary tumor, but was absent at recurrence despite a maintained Shh-like transcriptional signature (Morrissy et al., 2016). Interestingly, this type of subgroup stability may also occur in tumor evolution during metastasis. Specifically, a recent study using two non-overlapping cohorts of 12 and 19 paired, primary-metastatic tumors reported that MB subgroup affiliation remained stable between the primary and metastatic compartments, despite the potential for harboring different driver mutations (Wang et al., 2015). Therefore, pathway addiction is a theme that emerges not only in treatment with Smo antagonists, but also in metastatic and recurrent tumors treated with traditional approaches. Unfortunately, recurrent MB is almost uniformly fatal (Pizer et al., 2011). Together, these observations stress the
necessity of seeking treatments for such challenging cases and highlight the benefits of subgroup-based targeted therapies that could be applied upfront or in resistant cases.

**Primary cilia and reactivation of Hh signaling**

It has been suggested that primary cilia are essential for Gli processing (Roy, 2012). However, upon Hh binding to Ptch1, Gli2 and Gli3 accumulate in the cilium, leading to the hypothesis that Gli protein conversion to activated form is also regulated via transport or accumulation in the cilium. In fact, several studies have shown that IFT, the motor-driven transport that moves proteins along the cilium, is required for the production of GliAs and GliRs (Goetz and Anderson, 2010). Depending on developmental context, IFT mutants show loss of Hh phenotypes in some cell types and gain of Hh phenotypes in others. For example, GliAs are important in neural patterning and IFT mutant embryos show a loss of Hh signaling in the neural tube. Additionally, during early stages of normal cerebellum development, loss of primary cilia from GNPs prevents their expansion due to restricted Shh signaling (Chizhikov et al., 2007; Spassky et al., 2008). In contrast, GliRs have a central role in limb development, and IFT mutants can show phenotypes characteristic of GliR functional loss (Goetz and Anderson, 2010).

In tumors, cilia can also promote and oppose active Shh signaling, depending on context. Previous reports of primary cilia loss in BCC and Shh-dependent MB models suggest that cilia loss can block tumor initiation/proliferation driven by upstream Shh signaling (Ptch1 loss of Smo activation) or promote tumors with downstream activation by Gli2 amplification. We propose a variation of these models, in which pathway
reactivation and treatment resistance may arise with loss of primary cilia due to loss of the Gli2R but preservation of Gli2 activating functions. Our Gli2 knock-down data (Figure 3.4A-C) supports the importance of this transcription factor in resistant tumor survival, while the altered ratio of Gli2FL/Gli2R (Figure 3.4D) further implicates cilia-associated effects on Gli2. We propose a model in which Shh-dependent MB is transcriptionally driven by Gli2 activating functions, which are dominant to the lower levels of Gli2R produced in ciliated cells (Figure 5.1). However, if cilia are lost, Gli2R is no longer produced, and a cilia-independent mechanism allows for some Gli2-mediated transcriptional activity that drives tumor growth and resistance. Furthermore, additional genetic activation of the pathway can raise Shh signaling activity and proliferation, as described below.
Figure 5.1 Model of oncogenic Shh signaling in the context of primary cilia loss.

(A) In $Ptc^+/-$ tumors, constitutively active Smo promotes Sufu-Gli2 complex translocation into the primary cilium, where full-length Gli2 transcription factor (Gli$_{2FL}$) is released from Sufu and predominantly processed into Gli2 activator (Gli$_{2A}$), while low levels of Gli$_{2FL}$ are cleaved into Gli2 repressor (Gli$_{2R}$). The overall ratio of Gli$_{2A}$ to Gli$_{2R}$ favors activation of an oncogenic Shh transcriptional program. (B) Smo inhibition with LDE225 reduces Shh signaling by shifting the ratio of Gli$_{2A}$ and Gli$_{2R}$ to block Shh target gene transcription. (C) In cilia mutant tumors, Gli$_{2FL}$ is no longer processed into Gli$_{2R}$ due to loss of trafficking/function in the primary cilium. The majority of Gli$_{2FL}$ remains in complex with Sufu, but low amounts of Gli$_{2FL}$ are capable of maintaining low levels of Shh pathway activity regardless of upstream pathway inhibition by LDE225. (D) Genetic background of the tumor can alter levels of Shh pathway activity with cilia loss. For instance, loss of one copy of $Sufu$ in cilia mutant tumors is predicted to reduce the amount of Gli$_{2FL}$ that is sequestered in complex, and free more Gli$_{2FL}$ for activation of Shh target genes.
Figure 5.1 (continued)
Genetic background within the tumor may determine the level of Hh activity in the context of cilia functional loss

Among the three Ofd1 mutants from the transposon screen, we observed different levels of Shh activity, with clone LE2 exhibiting low Shh signal while clones GD10 and GG6 had higher levels. We hypothesize that the difference in Shh activity is due to other genetic changes in the mutant cells that directly impinge on Shh output. We have preliminary data indicating that reduced Sufu copy number (and thus reduced inhibition by Sufu) in GD10 and GG6 may explain the elevated Shh output compared to cilia mutants with both copies of Sufu (LE2 resistant clone). Such an inhibitory role for Sufu in the context of cilia loss is consistent with previous reports. Others have shown that Sufu retains its inhibitory role on GliA function in the absence of primary cilia (Jia et al., 2009). Furthermore, moderate knock-down (by 35-60%) of Sufu in cilia mutant fibroblasts is sufficient to release GliA inhibition. Importantly, partial loss of SUFU function may not be sufficient for resistance, since resistant BCCs with SUFU copy number loss had co-occurring alterations, such as GLI2 amplification (Sharpe et al., 2015). Loss of primary cilia may be another mechanism by which SUFU heterozygosity can mediate resistance.

Our results in genetically pure non-tumor cells provide further insight on the relationship between genetic background and Shh activity in the context of cilia loss. As expected, CRISPR-mediated depletion of Ofd1 in non-tumor cells abrogates ligand-induced pathway activation (Figure 2.5, Supplementary Figure S2.3). A similar trend of impaired response to Hh pathway agonists was shown for fibroblasts lacking cilia due to loss of KIF3a (Barakat et al., 2013). However, downstream activation of Shh signaling in
MEFs lacking Gli3 may help raise basal levels of activity that are maintained even after cilia loss. Our studies highlight the importance of genetic background in predicting whether there will be reactivation of Shh signaling (and possible resistance) in the context of cilia loss.

The role of HDACs in Hh-dependent tumors and resistance

Resistant BCC tumors have increased levels of GLI1 mRNA compared to untreated and sensitive tumors (Sharpe et al., 2015). Interestingly, we saw increased levels of HDAC2 mRNA in resistant BCC tumors, as well. The co-occurrence of GLI1 and HDAC2 over-expression could mean that HDAC2 is a direct transcriptional target of Shh activation, that HDAC2 promotes Shh target gene expression, or that HDAC2 is both a target and an activator of Shh transcription by functioning in a positive feedback loop like that described for HDAC1 (Canettieri et al., 2010). If HDAC2 is a transcriptional target of Gli proteins, it may cause resistance by mediating expression of other genes that promote tumor recurrence. The precise network of Gli target genes that mediates resistance is not known, and HDAC2 may be an early target gene that activates a resistance program. Whether the increased HDAC2 expression has consequences on tumor treatment is not yet known and something we will be exploring by targeted testing of HDAC2 as a candidate resistance gene.

If HDAC2 over-expression dampens or abrogates response to Smo inhibitors, it would suggest either that the anti-tumor effects of pharmacologic Smo inhibition require HDAC2 inhibition and/or HDAC2 over-expression can cause resistance to Smo inhibition. The latter would be an additional novel mechanism of resistance, beyond
those identified in Chapters 2, 3 and Appendix II. Additionally, it would be another variation of the theme in which reactivation of the Shh signaling axis results in resistance.

We did not find significant changes in expression of any other HDACs in resistant BCCs except for HDAC6 (data not shown). Previous reports have linked HDAC6 to promoting maximal Hh target gene expression, and have suggested targeting HDAC6 for treatment of SHH-MB (Dhanyamraju et al., 2015). Interestingly, HDAC6 is also involved in cilia disassembly during re-entry into the cell cycle. Thus, the increased HDAC6 levels in resistant human BCCs could promote resistance via enhanced Shh target gene expression and/or reduction of primary cilia (Izawa et al., 2015). In support of our observations, a recent study found that expression and activity of HDACs 1,2, and 6 were increased in tumors from mice expressing constitutively active Smo protein compared to wild-type cerebellum or cerebellum from Smo mutant mice that did not develop tumors (Lee et al., 2013).

Future work will be necessary to determine the mechanisms by which HDACs work with the canonical Shh signaling axis in tumors. Specifically, HDACs may be involved in initiation, maintenance, proliferation, and/or resistance of BCC and Shh-dependent MB. Additionally, combination treatment with Smo antagonists and HDAC inhibitors may help prevent the emergence of resistance.

**HDAC inhibitors as treatment**

Early reports have shown anti-tumor activity of different HDAC inhibitor treatments in mouse models of MB carrying loss of *Ptch1* function and gain-of-function
mutations in Smo (Ecke et al., 2009; Spiller et al., 2006). Unlike these reports, we have more comprehensively evaluated the potential for HDAC inhibitors by screening over 70 such compounds, spanning several chemical classes and showing that the majority of them do not show anti-tumor activity in Shh-dependent MB cells. We also tested several of these inhibitors in different cell lines, and in particular cells with clear mechanisms of resistance to current targeted therapies. Our work thus provides insight into how we can differentiate between effective early-stage small molecules that can be further developed into targeted HDAC inhibitors. For example, since we see indication that HDAC1 knock-down is effective in reducing tumor cell proliferation, it may be possible to use the chemistry of those inhibitors that work to make more specific antagonists to HDAC1. Targeting only HDAC1 or HDAC2 may also be helpful in reducing general toxicity to healthy tissues, as these two enzymes are known to play redundant roles. Mice lacking both HDAC1 and HDAC2 (but not one or the other) in neuronal precursors show abnormal brain development attributed to failure of neuronal precursors to differentiate into mature neurons and excessive cell death (Montgomery et al., 2009). If resistant tumors rely on increased HDAC2 function, it may provide a therapeutic window in which targeting HDAC2 specifically will be effective against tumors without damaging non-tumor cells.

**Conclusion**

In this set of studies, we present a genome-wide screen for resistance mechanisms to targeted Smo inhibition in Shh-dependent MB. We identify and validate Ofd1 as a previously unrecognized resistance gene. We also show that primary cilia
loss in Ofd1 mutant cells, as well as in cells with loss of other cilia genes, contributes to resistance by reactivating Shh signaling mediated by the Gli2 transcription factor. Evidence from an in vivo study as well as from resistant human BCC samples suggests that primary cilia loss is a feature of resistant tumors. Finally, we present the results of a high-throughput small molecule screen and show that HDAC inhibitors can reduce survival of several MB cells lines, including those which are resistant to Smo antagonists. These studies contribute to an understanding of oncogenic signaling in Shh-dependent tumors and propose important considerations for targeted treatment approaches.
Appendix I: Supplementary Figures and Tables
Supplementary Figure S2.1 Copy number analysis of screen mutants identifies loss of Sufu and amplification of Gli2. Transposon screen mutants were analyzed for genomic copy number changes previously associated with resistance to Smo inhibitors. SINE-1 normalized copy number was calibrated to SMB21 parental cell DNA (A) or mouse genomic DNA (B), as explained in the methods. Mutants with transposon insertions in Sufu or Ofd1 (Figure 2.3) are highlighted in red or green, respectively. Bars represent the mean of n=3-4 independent experiments.
Supplementary Figure S2.2 Hh target gene expression in *Sufu* and *Ofd1* screen mutants resembles expression in parental cells. Relative expression levels of known Shh target genes in *Sufu* and *Ofd1* screen mutants are consistent with parental cells. In contrast, parental cells treated with the Smo inhibitor LDE225 (LDE) show reduction in Shh target gene expression. Treatment of parental cells with LDE highlights the Shh-dependent gene signature by gene expression changes.
Supplementary Figure S2.3 Ofd1 depletion in MEFs abrogates ligand-dependent activation of Shh signaling. (A) CRISPR/Cas9-mediated depletion of Ofd1 in wild-type (WT) or homozygous mutant MEF cells abrogates SAG-induced activation of Shh signaling, as measured by Gli1 mRNA normalized to GAPDH mRNA levels. (B) Depletion of Sufu increases basal levels of Gli1 mRNA compared to EGFP depletion controls.
Supplementary Figure S2.3 (continued)

A.

WT

p53⁻/⁻

Gli2⁻/⁻

Gli1 mRNA

VEH

SAG

Ptch1⁻/⁻

Gli3⁻/⁻

Gli1 mRNA

VEH

SAG

B.

Gli1 mRNA

WT

p53⁻/⁻

Ptch1⁻/⁻

Gli2⁻/⁻

Gli3⁻/⁻
Supplementary Figure S2.4

Some *Sufu* and *Ofd1* resistant clones cannot be rescued for drug sensitivity with Flp. (A-C) Resistant clones with transposon insertions in *Ofd1* (A,B) or *Sufu* (C) cannot be rescued for sensitivity to LDE225 (LDE) by lentiviral delivery of Flp. Relative survival was assessed at 0, 10, 100 and 1000 nM LDE. One-way ANOVA with Dunnett’s correction for each treatment condition versus 0 nM LDE (n= 7, 3, and 4 independent experiments for GD10, GG6, and DG8, respectively, mean ± SEM).
Supplementary Figure S2.5 Addition of Sufu or Flp can rescue some resistant mutants for sensitivity to LDE225. All 27 resistant clones that were propagated in culture were tested for LDE225 sensitivity rescue by lentiviral delivery of Sufu or Flp. Resistant clones with transposon insertions in *Sufu* and *Ofd1* are highlighted in red and green, respectively. A rescue by Sufu suggests loss of Sufu function as the mechanism of resistance. Resistant clone LF2 was not tested with Sufu rescue. A rescue by Flp correlates transposon insertion with resistance. If Flp successfully removes the transposon(s), the cells become sensitive to puromycin. Relative survival was assessed at 0, 10, 100 and 1000 nM LDE or 1 mg/mL puromycin. The heatmap represents the mean of n=1-7 independent experiments for each resistant clone.
Supplementary Figure S2.6 Depletion of Ofd1 and Sufu causes resistance in two independently established medulloblastoma cell lines. (A) CRISPR/Cas9-mediated depletion of Ofd1 and Sufu in the SMB55 cell line results in functional resistance to LDE225 (LDE). Relative survival was assessed at 0, 10, 100 and 1000 nM LDE. (B) Depletion of Ofd1 and Sufu in the SMB56 cell line also results in resistance to LDE. Statistical analysis by one-way ANOVA with Dunnett’s correction for each treatment condition versus 0 nM LDE, *p<0.01, **p<0.005, ***p<0.001, ++p<0.0001 (n = 3 independent experiments, mean ± SEM).
Supplementary Figure S2.7. Summary of transposon screen resistant clones and follow-up characterization. All resistant clones fail to respond to LDE225 by survival assay and Gli1 protein levels. Thirteen resistant clones had transposon insertions in *Sufu* (S) and three had insertions in *Ofd1* (O). Full or partial Sufu loss was confirmed by immunoblot for all thirteen clones with transposon insertion in this gene, in addition to six clones that lost Sufu protein but did not have transposons in the *Sufu* gene. Copy number analysis indicated Gli2 amplification in resistant clone LB16. Rescue experiments with *Sufu* over-expression or Flp-mediated transposon removal are summarized here and in Supplementary Figure S2.5.
Supplementary Figure S3.1

Depletion of Kif3a reduces LDE225 response in SMB55 and SMB56 medulloblastoma cells. (A) CRISPR/Cas9-mediated depletion of Kif3a in the SMB55 cell line opposes inhibition by LDE225 (LDE). Relative survival was assessed at 0, 10, 100 and 1000 nM LDE. (B) Depletion of Kif3a in the SMB56 cell line results in resistance to LDE. Statistical analysis by one-way ANOVA with Dunnett’s correction for each treatment condition versus 0nM LDE, *p<0.05, **p<0.005, ***p<0.001, ++p≤0.0001 (n = 3 independent experiments, mean ± SEM).
Supplementary Figure S3.2 Knock-down of Smo preferentially reduces survival of parental cells compared to Smo inhibitor resistant mutants. (A) shRNA targeting Smo reduces target mRNA levels normalized to GAPDH and an shRNA targeting Luc. (B) Knock-down of Smo reduces survival of parental cells more readily than DG8 (Sufu mutant) and GD10 (Ofd1 mutant) cells. Statistical analysis done by two-way ANOVA, with Bonferroni’s correction, *p<0.05, **p<0.005; ***p<0.0001 (n=3 independent experiments, mean ± SEM).
Supplementary Figure S3.3 Clinically resistant BCCs have increased cilia gene mutations. (A) Vismodegib resistant clinical BCC samples show significantly increased cilia gene mutations compared to untreated samples. The same analysis was done as in Figure 3.5D, except that SMO mutations were excluded here. Mann-Whitney test, p<0.05 (n= 48 untreated and 11 resistant samples). (B) Resistant BCCs have significantly more recurrently mutated cilia genes compared to untreated patients. The same analysis was done as in Figure 3.5F, except that SMO mutations were excluded here. Mann-Whitney test, p<0.0001 (n= 303 cilia genes).
Supplementary Figure S4.1 Summary of responses in dose screen. SMB21 and Gli2ΔN cell lines were screened with 79 small molecules at 5 doses each: (A) 0.12-10 μM dose range for GPCR targets and (B) 0.0625-1 μM dose range for kinase and epigenetic targets. Survival was normalized to DMSO-treated cells.
Supplementary Figure S4.2 JNJ-26481585 does not induce significant cell death in the cortex. TUNEL-positive cells in the cortex from the same slice cultures as shown in Figure 4.5C were not significantly increased with the HDAC inhibitor JNJ-26481585 compared to DMSO and LDE225 controls. One-way ANOVA with Tukey correction (n=4 independent experiments with 3-4 slices per treatment group and 3 images per slice, mean ± SEM).
Supplementary Figure S4.3 Knock-down of Smo or HDAC1 reduces Gli1 and HDAC1 protein levels. Lentiviral shRNA (shHdac1-1, shHdac1-4 and shHdac1-5) reduced Hdac1 but not Hdac2 levels. Smo knockdown reduced Hdac1 and Hdac2 levels.
**Supplementary Table S4.1 HDAC inhibitors from primary screen.** The primary screen included 76 small molecules that can target HDACs. The 9 HDAC inhibitors that came up as hits in the primary screen are highlighted.

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Appendix II: RAS/MAPK Activation Drives Resistance to Smo Inhibition, Metastasis, and Tumor Evolution in Shh Pathway-Dependent Tumors
Introduction

Sonic Hedgehog (Shh) signaling plays a critical role in growth and patterning during development, and aberrant activation of Shh signaling is implicated in several cancers (1). Germline mutations that activate Shh signaling predispose to basal cell carcinoma (BCC) and medulloblastoma in humans and mice, whereas somatic mutations in Shh components are frequently observed in such tumors (2, 3).

Signaling is initiated when Shh, a secreted protein, binds its receptor, Patched (Ptch). In the absence of Shh, Ptch suppresses activity of Smoothened (Smo). When Shh binds Ptch, Smo initiates a signaling cascade, which inactivates the tumor-suppressor Suppressor of Fused (Sufu), and activates Gli transcription factors. Gli target genes include Gli1, Peh, Nmyc, and Cyclin D1 (Cyclin D1), Small-molecule antagonists of Smo, including Vismodel (DCC-0449; Roche), Sonidegib (LDE225; Novartis), and XL-139 (Bo2/Tasilina), promise promising targeted therapy for BCC and medulloblastoma (1, 4–7), and are in clinical trials or in use for these indications.

Despite initial success of Smo inhibitors, long-term efficacy is limited by pre-existing or acquired drug resistance (8, 9). Studies of other cancers indicate that both cell-autonomous mutations and microenvironment-derived factors contribute to therapeutic resistance (10). Amplification of Gli2 and point mutations in Smo that prevent drug binding have been reported to cause resistance in preclinical and clinical studies (4, 5, 11). Increased activation of PI3K, aPKC- and microenvironment-driven factors contribute to therapeutic resistance (10). Additional mechanisms of resistance are likely to arise in clinical practice, and must be understood to develop more effective therapeutic strategies for Shh-dependent tumors.

RAS/MAPK Activation Drives Resistance to Smo Inhibition, Metastasis, and Tumor Evolution in Shh Pathway–Dependent Tumors

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Abstract

Aberrant Shh signaling promotes tumor growth in diverse cancers. The importance of Shh signaling is particularly evident in medulloblastoma and basal cell carcinoma (BCC), where inhibition targeting the Shh pathway component Smoothened (Smo) show great therapeutic promise. However, the emergence of drug resistance limits long-term efficacy, and the mechanisms of resistance remain poorly understood. Using new medulloblastoma models, we identify two distinct paradigms of resistance to Smo inhibition. Sufu mutations lead to maintenance of the Shh pathway in the presence of Smo inhibitors. Alternatively activation of the RAS–MAPK pathway circumvents Shh pathway dependency, drives tumor growth, and enhances metastatic behavior. Strikingly, in BCC patients treated with Smo-inhibitor, squamous cell cancers with RAS/MAPK activation emerged from the antecedent BCC tumors. Together, these findings reveal a critical role of the RAS–MAPK pathway in drug resistance and tumor evolution of Shh pathway–dependent tumors.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).
the MAPK pathway also becomes activated after vismodegib treatment as Shh-dependent basal cell cancer transitions to squamous cell cancer resistant to Smo inhibitors. Together, these results indicate thatactivation of the Shh pathway or interactions between Shh and MAPK pathways can alter tumor behavior and therapeutic responses. Therefore, future treatments must consider these distinct mechanisms of tumor evolution.

Materials and Methods

Detailed description is given in Supplemental Materials.

Animals

All experimental procedures were done in accordance with the NIH guidelines and approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee.  
Pdx1Cre+/− mice (The Jackson Laboratory, of 2),  
uhc mice (Charles River Laboratories).

Human studies

All human subjects work was reviewed by the Institutional Review Board Committees of Brigham and Women’s Hospital and Dana-Farber Cancer Institute, University of Calgary, and Stanford University for appropriate use, that informed consent was obtained from all subjects when required, and appropriate waiver of consent requirements was obtained for minimal risk studies.

SMB cell culture

Shh-subtype medulloblastoma (SMB) cells were cultured as neurospheres in DMEM/F12 media (2% B27, 1% Pen/Strep).

Cell survival assays

SMB cells in 96-well plates (5 × 10^3 cells/well) were incubated for 72 hours in LDE225, vismodegib, TMZ, or ATO, or for 120 hours in BRM120, BEZ235, PD325901, or CI-1040. Viability was measured using CellTiter 96 Aqueous One Solution (Promega). The microarray data accession number is GSE69359.
Tuj1, cerebellar marker Zic1, cell proliferation marker Ki67, and Math1(Atoh1), a hallmark of Shh-subtype medulloblastoma (Fig. 1A and B). Importantly, SMB cells exhibit constitutively activated Shh signaling, as Shh signaling components and target genes Gli1, Gli2, Boc, Ccnd1, Nmyc, and SFRP1, are highly expressed in SMB cells and in primary medulloblastoma from Ptch1/2−/− mice (Fig. 1B). To compare SMB cells with human medulloblastoma, we performed gene-expression analysis of SMB cells, in vivo primary medulloblastoma, cerebellum, analyzed using signature profiles of human medulloblastomas (Fig. 1C).
medulloblastoma ([WNT, SHH, group C, group D; ref. 18]. Similarity to each subtype for each sample is defined by the "signature score," which quantitatively measures similarity of gene-expression patterns to predefined signature genes. Subtype signature score indicates that SMB cells and in vivo primary medulloblastomas both closely resembled SHH-subtype medulloblastoma (Fig. 1C). We used a second algorithm, agreement of differential expression (AGDEX; 19), to compare SMB cells with human medulloblastoma. AGDEX analysis also indicates that both Ptch+/− primary medulloblastomas and SMB cells exhibit the highest agreement with human SHH subtype (Supplementary Fig. S1F). Notably, activated Shh signaling in SMB cells is uniquely sensitive to Smo inhibitors, as demonstrated by reduced Gli1 mRNA and protein following treatment with Smo inhibitor LDE225 (Fig. 1D and E). Importantly, proliferation and survival in cells treated with LDE225 (Supplementary Fig. S1G) recapitulate in vivo results. (Fig. 1F). We used a second algorithm, agreement of differential expression (AGDEX) analysis to test resistance of engineered SMB cells to LDE225. To test resistance of engineered SMB cells, we used SMB cells to test three groups of candidates. The first group includes key Shh pathway components downstream of Smo: Sufu, Gli1, Gli3, and Nmyc. We used the juggerknot transposon system to overexpress Gli1, Gli3, or Nmyc, or used shRNA to knockdown Sufu. Unlike Gli2, expression of Gli1, Gli3, or Nmyc in SMB cells cannot bypass Smo inhibition, reactivates Shh signaling, or promote proliferation and survival in cells treated with LDE225 (Supplementary Fig. S2D and S2F). Consistent with previous publications of medulloblastomas (27, 28), neither expression of wild-type nor overexpression of stabilized MYC, MYC(T58A), confer resistance to LDE225 in SMB cells (Fig. S3A and B). Consistent with the key role of Sufu, several of the resistant tumors that arose spontaneously from subcutaneously implanted SMB cells following treatment with LDE225 showed drastic reduction of Sufu expression (Fig. 3D). To identify possible treatment for tumors that are resistant to Sufu loss, we tested an inactivating NRAS mutation (29), an inhibitors of Gli2, expression of Gli2, in SMB cells cannot bypass Smo inhibition, reactivates Shh signaling, or promote proliferation and survival in cells treated with LDE225 (Supplementary Fig. S2D and S2F). Consistent with previous publications of medulloblastomas (27, 28), neither expression of wild-type nor overexpression of stabilized MYC, MYC(T58A), confer resistance to LDE225. To test resistance of engineered SMB cells to LDE225, we used three groups of candidates. The first group includes key Shh pathway components downstream of Smo: Sufu, Gli1, Gli3, and Nmyc. We used the juggerknot transposon system to overexpress Gli1, Gli3, or Nmyc, or used shRNA to knockdown Sufu. Unlike Gli2, expression of Gli1, Gli3, or Nmyc in SMB cells cannot bypass Smo inhibition, reactivates Shh signaling, or promote proliferation and survival in cells treated with LDE225 (Supplementary Fig. S2D and S2F). Consistent with previous publications of medulloblastomas (27, 28), neither expression of wild-type nor overexpression of stabilized MYC, MYC(T58A), confer resistance to LDE225. To test resistance of engineered SMB cells to LDE225, we used three groups of candidates. The first group includes key Shh pathway components downstream of Smo: Sufu, Gli1, Gli3, and Nmyc. We used the juggerknot transposon system to overexpress Gli1, Gli3, or Nmyc, or used shRNA to knockdown Sufu. Unlike Gli2, expression of Gli1, Gli3, or Nmyc in SMB cells cannot bypass Smo inhibition, reactivates Shh signaling, or promote proliferation and survival in cells treated with LDE225 (Supplementary Fig. S2D and S2F). Consistent with previous publications of medulloblastomas (27, 28), neither expression of wild-type nor overexpression of stabilized MYC, MYC(T58A), confer resistance to LDE225. To test resistance of engineered SMB cells to LDE225, we used three groups of candidates. The first group includes key Shh pathway components downstream of Smo: Sufu, Gli1, Gli3, and Nmyc. We used the juggerknot transposon system to overexpress Gli1, Gli3, or Nmyc, or used shRNA to knockdown Sufu. Unlike Gli2, expression of Gli1, Gli3, or Nmyc in SMB cells cannot bypass Smo inhibition, reactivates Shh signaling, or promote proliferation and survival in cells treated with LDE225 (Supplementary Fig. S2D and S2F).
RAS Drives Drug Resistance and Tumor Evolution in Shh Tumors

**Figure 2.** SMB cells provide a model to study resistance to Smo inhibition. A, survival analysis of SMB21 cells expressing GFP (control), Smo mutants, or Gli2DN treated with indicated LDE225 concentrations (72 hours; mean ± SEM; n = 4). B, Shh signaling was analyzed by immunoblot for Gli1 in SMB21 cells expressing wild-type or mutant Smo, treated with DMSO or 1 mmol/L LDE225 for 24 hours. C, SMB21 cells expressing Smo(WT) retain LDE225 responsiveness in vivo; SMB21 cells expressing Smo(D477G) or Gli2DN initiate resistant tumors; tumor volume over time; mean ± SEM; n = 5.

Supplementary Fig. S3B and S3D. HRAS(G12V) and BRAF(V600E) also cause resistance to other Smo inhibitors LEQ506 and vismodegib, indicating that this effect is generalizable for Smo antagonists (Supplementary Fig. S3E and S3F). SMB(HRAS) cells subcutaneously transplanted in nude mice were resistant to treatment with Smo inhibitors (Fig. 4D). Furthermore, MAPK activation is greater in SMB tumors that spontaneously develop resistance to Smo inhibitors following treatment with LDE225 than in vehicle-treated, sensitive tumors (Fig. 4E). Taken together, these data indicate that activation of RAS/MAPK provides a novel way for cells to evade Smo inhibition.

Surprisingly, HRAS(G12V) does not confer resistance by reactivating Shh signaling downstream of Smo (Fig. 5A). Instead, HRAS(G12V) suppresses Shh signaling in SMB cells, as expression of multiple Shh pathway targets and components are down-regulated in SMB(HRAS) cells (Fig. 5A–C; Supplementary Fig. S4A–S4D). Notably, Math1(Atoh1), a hallmark of Shh-subtype medulloblastoma that is not a direct target of SHH signaling, is decreased in SMB(HRAS) cells. These results suggest that HRAS renders SMB cells independent of Shh-signaling for growth, and thereby causes resistance.

RAS is normally regulated by upstream GFs and receptor tyrosine kinases, and so receptor activation might mimic the effects of HRAS in SMB cells. During normal cerebellar GCP development, GF, such as bFGF, antagonize Shh pathway activity and promote differentiation (29, 30). When SMB cells were exposed to GFs that are common components of stem cell media (bFGF and EGF; 20 ng/mL) both PI3K–AKT and RAS–RAF-MAPK pathways were activated, Shh signaling was suppressed, and SMB cells became resistant to LDE225 (Fig. 5D–G). We tested the GFs individually, and found that bFGF, not EGF, suppresses Shh signaling and causes resistance (Supplementary Fig. S4E and S4F). Together, these results demonstrate that sustained activation of FGF/RAS/RAF signaling enables Shh-subtype medulloblastoma to grow in a Shh pathway-independent manner.
Loss of Sufu confers resistance to Smo inhibition. A, relative survival for SMB21, SMB55, SMB56 with shRNA knockdown of Sufu treated with indicated LDE225 concentrations (72 hours; mean ± SEM; n = 3). B and C, genomic copy number of Sufu gene (SUFU) and β-actin (Actin) in sensitive and resistant tumors determined by quantitative PCR; E, relative survival for SMB21 and Sufu-luc knockdown cells treated with ATO (72 hours; mean ± SEM; n = 3).

FGF/RAF-mediated resistance to Smo inhibitors is reversible
To investigate whether FGF/RAF/MEK pathway activation is required for SMB cells to both develop and maintain resistance to Smo inhibitors, we removed oncogenic HRAS from SMB(HRAS) cells using lentiviral delivered Flp to cleave FRT sites within the transposon (Supplementary Fig. S5A and S5B). Removal of HRAS decreases Erk phosphorylation and restores Shh signaling activity and susceptibility to Smo inhibitors (Fig. 6A–C). Similarly, cells resistant to Smo inhibition due to prolonged BCF treatment regained Shh signaling activity and susceptibility to Smo inhibitors when BCF was removed from media for prolonged time periods (Fig. 6D and E). Together, these data indicate that prolonged activation of FGF/RAF/MEK signaling both initiates and maintains Shh-signaling independence and resistance to Smo inhibitors.

To identify therapeutic approaches for treating SMB cells resistant to Smo inhibitors, we tested PDK1 and MEK pathway inhibitors. Although HRAS cells showed similar sensitivity to Smo parental cells to PDK1 inhibitors BE22135 and BRR31220, they were much more sensitive to MEK inhibitors CI-1040 and PD325901. Thus, MEK inhibitors provide therapeutic treatment for resistant tumors driven by activation of FGF/RAF signaling (Fig. 6F and G; Supplementary Figs. S5C–S5E, S6).

RAS/MEK activation alters characteristics of Shh pathway–dependent tumors
Morphologically, SMB(HRAS) cells exhibit a dramatically different appearance from SMB parental cells. SMB parental cells are small, with little cytoplasmic material, whereas SMB(HRAS) cells appear larger with extended cellular processes (Supplementary Fig. S7). Immunohistochemical characterization revealed that SMB(HRAS) cells are proliferative, as indicated by Ki67, and poorly differentiated, as indicated by Nestin, a stem cell/progenitor marker (Fig. 7A).

The striking morphologic differences in SMB(HRAS) cells suggest that they may be more motile than parental cells. Indeed, these cells were more invasive when tested in a Matrigel invasion assay (Fig. 7B). Interestingly, when SMB(HRAS) cells were s.c. injected in nude mice, they initiated resistant tumors, and also generated lung metastases in 2 of 9 mice. Metastases were never found in 8 mice injected with SMB cells (Fig. 7C and D). Together, these data indicate that activation of RAS/MEK increases tumor invasiveness.

Clinical observations from a rare set of matched primary and metastatic lesions from an individual patient provide additional evidence that RAS activation promotes metastasis in medulloblastoma, as the primary lesion exhibits low level of MEK activation, whereas the metastatic frontal lobe lesion exhibits robust MEK activation (Fig. 7E and F). In human primary Shh-subtype medulloblastoma, most areas are predominantly negative for MEK activation (Supplementary Fig. S6; Table S2); however, in one tumor, cancer cells in the perivascular niche were strikingly positive for ERK phosphorylation (Supplementary Fig. S6D) whereas in a desmoplastic/nodular medulloblastoma, ERK phosphorylation was elevated in perivascular regions (Supplementary Fig. S6B). Both tumors exhibited regions with high FGF immunostaining (Supplementary Fig. S6B and S6D). Cells with MEK activation within human Shh subtype medulloblastomas may generate resistant tumors when challenged by Smo inhibitors.

Vismodegib treatment of BCC engenders RAS/MEK-dependent tumors
Smo inhibitor vismodegib is approved for clinical use for patients with advanced BCC, but is not yet approved for...
Figure 4. RAS/MAPK signaling confers resistance to Smo inhibitor. A–C, relative survival for SMB21 cells expressing candidate genes treated with LDE225 (72 hours). HRAS(G12V), BRAF(V600E), not PIK3CA(H1047R) or myristoylated AKT, confer resistance; mean ± SEM; n > 5. A', elevated phospho-Erk in SMB21 cells expressing HRAS(G12V) or BRAF(V600E). B' and C', elevated phospho-AKT in SMB21 cells expressing PIK3CA(H1047R) or AKT(Myr). D, SMB21(HRAS) cells initiate resistant tumors in vivo; tumor volume over time; mean ± SEM; n = 5. (Experiments were performed concurrently with Fig. 2C, the same SMB21 control shown here and Fig. 2C.) E, phospho-Erk in sensitive and resistant tumors from engrafted SMB21 cells mean ± SEM; *, P < 0.05, unpaired Student t test.
medulloblastoma. Among BCC patients, approximately 21% that initially respond subsequently develop tumors resistant to this inhibitor (31). In some cases, posttreatment resistant tumors exhibit characteristics of squamous cell carcinoma (SCC; refs. 32–36). We analyzed three patients who developed SCC at the site of the antecedent BCC tumor following treatment with vismodegib. Posttreatment resistant tumors display low level of Gli1 and high level of phospho-ERK, suggesting upregulated RAS/MAPK and downregulated Shh signaling (Fig. 7G). To determine whether SCCs that develop following vismodegib are derived from the antecedent BCC in the same location, we analyzed patient-matched normal tissue or blood, and pre- and post-relapse tumor samples by exome sequencing with germline and dbSNP variants removed. Tumors we assayed initially responded to vismodegib before acquiring resistance to the inhibitor. Strikingly, 91% of genetic variations (n = 1248) in the SCC sample were shared between pretreatment BCC and posttreatment SCC, whereas only 3% and 6% of somatic genetic variations (n = 43 and n = 84) were unique to the SCC or shared with patient-matched normal sample, respectively, suggesting that the SCC arose from the BCC (Fig. 7H). These results suggest that tumors can evolve from Shh pathway-dependent BCC to the RAS-MAPK pathway-dependent SCCs, and thereby develop resistance to Smo inhibitors.
RAS Drives Drug Resistance and Tumor Evolution in Shh Tumors

Discussion

The studies presented here introduce a set of Shh pathway-dependent medulloblastoma cell lines (SMB) and identify two distinct mechanisms of therapeutic resistance to Smo inhibitors. Loss of Sufu drives resistance to Smo inhibition by activating downstream Shh signaling. Alternatively, activation of RAS/ERK signaling, either due to new mutations or to
microenvironmental factors, constitutes a novel mechanism of resistance that circumvents Shh pathway dependence in a growing tumor.

**SMB cells as a model for Shh-subtype medulloblastoma**

Cell lines that faithfully model the cancer from which they are derived provide important tools for studying disease mechanism.
and discovering novel therapies. Investigation of medulloblastoma biology has been limited by the lack of stable lines that are tumorigenic and Shh pathway dependent. Most established medulloblastoma lines, are adherent cell cultures maintained in serum-containing media, and do not depend on the Shh pathway for growth and survival (6, 14). Current protocols can only culture freshly isolated tumor cells for a short time before key characteristics of Shh-subtype medulloblastomas are lost. Here, we present Shh pathway–dependent medulloblastoma lines (SMB) that can be used as effective and faithful in vitro models to study Shh-subtype medulloblastoma.

The protocol that enabled development of SMB lines is a modified version of neural stem cell culture methods. Key aspects include growing cells as nonadherent spheres and eliminating serum, FGF and bFGF from media. Generation of tumor spheres is commonly used to enrich for cancer stem-like cells (37). Indeed high-grade gliomas can be perpetuated as neurospheres by maintaining cells in neural stem cell media with EGF and bFGF (37, 38). However, these conditions do not maintain tumorigenicity and Shh pathway dependency of medulloblastoma cells (39), as FGF signaling has an antagonistic effect on Shh signaling (29, 30, 40). Instead, medulloblastoma neurospheres from Pch−/− mice cultured without exogenous EGF or bFGF generate Shh-subtype medulloblastoma cell lines (SMB) that are tumorigenic, maintain markers of Shh-subtype medulloblastoma and remain dependent on Shh pathway activity even after many passages in vitro.

A previous study isolated rare lines from medulloblastomas of Pch−/−; p53−/− mice (41). We observe distinct modes of p53 signaling dysregulation in each SMB line. Because all three lines are derived from Pch−/−; p53−/− mice, mutations or inactivation of p53 signaling may have developed during primary medulloblastoma tumorigenesis in each individual animal. In human medulloblastoma, the importance of p53 has become increasingly apparent. Among human medulloblastomas, p53 mutations were detected in 13% to 21% of Shh-subtype MBs (21, 22). We conclude that SMB cells offer a faithful model for investigating the Shh pathway in medulloblastoma, and facilitate high-throughput drug testing and large-scale functional screens. In the future, a similar approach might enable generation of human Shh-subtype medulloblastoma and remain dependent on Shh pathway activity even after many passages in vitro.

Mechanisms of resistance to Smo inhibition
Several Smo inhibitors show promise in preclinical and clinical studies. Vismodegib was the first drug of this class approved by the FDA to treat BCCs. In one study, 6 of 28 BCC patients treated with vismodegib developed resistance to Smo inhibition (31). Here, we demonstrate that p53 mutation can occur after treatment with Smo inhibition, and cause secondary resistance by activating Shh signaling downstream of ErbB. As loss of Sufu generates medulloblastomas that never respond to Smo inhibition (21, 42), this finding provides proof of principle that clinically relevant resistance mechanisms can be studied in SMB cells.

Data that overexpression of Gli1 or Nmyc does not confer resistance to Smo inhibition may seem surprising. However, Gli2 is the primary activator of the Shh signaling pathway in GCP development and medulloblastoma, whereas Gli3 is not essential (43, 44). In preclinical and clinical settings, amplification and constitutive activation of Gli2 generate Shh-subtype medulloblastoma resistant to Smo inhibition (5, 12, 21). In a recent study of human Shh-subtype medulloblastoma (n = 133), 10 cases of Gli2 amplification were identified, but no cases of Gli3 amplification were seen (21). Therefore, our results with overexpression of Gli transcription factors are consistent with clinical observations. In contrast, Nmyc amplification is reported in Shh-subtype medulloblastoma that do not respond to Smo inhibition (21). Although we cannot exclude the possibility that higher expression achieved by other means might confer resistance, our results suggest that other genetic changes may be needed in conjunction with Nmyc for tumors to grow in the presence of Smo inhibitors.

An important finding of this study is identification of a Shh pathway–independent mechanism of resistance. Smo-mediated resistance involves shifting oncogenic addiction to a second oncogenic pathway. De novo mutations or a microenvironment with abundant GF can stimulate RAS/MAPK signaling, eliminate Shh pathway dependency, and cause resistance in medulloblastoma. Indeed, in human medulloblastoma, components of the RAS–RAS–MAPK pathway are often overexpressed (27), and epigenetic inactivation of RAS association domain family 1A (RAF1) tumor-suppressor gene is frequently observed (45, 46). Although de novo RAS mutations have not been detected in primary human medulloblastoma, such mutations might be favored following treatment with Smo inhibition (28), as mutations that confer resistance are often only detected following treatment with targeted therapies (11, 47). Strikingly in our studies, xenografts with spontaneous resistance to Smo inhibitors display activation of ERK signaling in vivo. Thus, our data indicate that GF stimulation, genetic or epigenetic changes affecting the RAS–RAF–MEK pathway should be assessed in patients that develop resistance to Smo inhibitors.

In addition to intrinsic mutations in tumor cells, tumor microenvironment may alter drug efficacy (48). Our study suggests that microenvironments with abundant GF could provide protective niches for cells exposed to Smo inhibitors. We show that in human medulloblastoma, ERK activation occurs in locations adjacent to blood vessels or in perinodular spaces. Recent work suggested that stromal production of placental GF (PlGF) in human medulloblastoma promotes cancer cell survival by activating the MAPK cascade (49). Thus, paracrine PlGF-mediated RAS/MAPK signaling could also attenuate efficacy of Smo inhibitors.

Cross-talk between Shh and FGF/RAS signaling has been widely recognized during organogenesis in multiple tissues (50, 51). Depending on biologic context, interactions between FGF/RAS and Shh pathways can be synergistic or antagonistic. In cerebellar GCPs and Pch−/− medulloblastoma cells, acute bFGF treatment suppresses Shh signaling and proliferation, and concomitantly promotes cell differentiation (29, 30). Similarly, oncogenic RAS can block Shh signaling in NIH3T3 cells and pancreatic cancer models (52). Here, we again observe an antagonistic relationship between Shh and FGF/RAS signaling in SMB cells. Importantly, however, this process does not promote terminal differentiation; instead tumor cells remain proliferative and tumorigenic.

RAS/MAPK signaling in metastasis and tumor evolution
Strikingly, RAS/MAPK activation alters multiple characteristics of Shh-dependent tumors. Morphologic and transcriptional profiles of Shh(HRAS) cells differ from Shh cotelan cells although Shh cells are small with classic medulloblastoma histology. SMB(HERAS) cells display an extended morphology, are more invasive in vitro and metastasize faster in vivo (53). RAS/MAPK signaling may also alter Shh signaling. Cross-talk between Shh and RAS/MAPK signaling promotes Gli1 expression and Shh signaling in vivo (54, 55). In a recent study of human medulloblastoma, RAS/MAPK resistance involves shifting oncogenic addiction to a second oncogenic pathway. De novo mutations or a microenvironment with abundant GF can stimulate RAS/MAPK signaling, eliminate Shh pathway dependency, and cause resistance in medulloblastoma. Indeed, in human medulloblastoma, components of the RAS–RAS–MAPK pathway are often overexpressed (27), and epigenetic inactivation of RAS association domain family 1A (RAF1) tumor-suppressor gene is frequently observed (45, 46). Although de novo RAS mutations have not been detected in primary human medulloblastoma, such mutations might be favored following treatment with Smo inhibition (28), as mutations that confer resistance are often only detected following treatment with targeted therapies (11, 47). Strikingly in our studies, xenografts with spontaneous resistance to SM inhibitors display activation of ERK signaling in vivo. Thus, our data indicate that GF stimulation, genetic or epigenetic changes affecting the RAS–RAF–MEK pathway should be assessed in patients that develop resistance to Smo inhibitors.

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and more metastatic in vivo. Comparison of Shh-subtype primary medulloblastoma and matched metastatic lesions from the same person, revealed high level of phosphorylated ERK1/2 in metastases. Consistent with our findings, ectopic expression of Eaz (embryonic stem-cell-enriched Ra), which is structurally similar to ERK (53), increases leptomeningeal metastasis in models of Shh subtype medulloblastoma, and these metastatic cells differ genetically and epigenetically from primary tumor cells (54, 55). Together, these data indicate that RAS activation results in resistance to Smo inhibitors and alter tumor characteristics.

Clinical studies of resistant BCC also suggest a role of RAS/MEK signaling in tumor evolution. Several studies have reported occurrences of SCCs during treatment of BCC with vismodegib (19–26). Sequencing of matched pre- and posttreatment skin tumor samples supports the hypotheses that BCC tumor acquires RAS/ERK and/or PI3K resistance by which Shh pathway-dependent tumor cells evolve and escape Shh signaling dependence. Future studies are required to assess the prevalence of this resistance mechanism in patients.

Disclosure of Potential Conflicts of Interest
A.J.S. Chang reports receiving other commercial research support and is a consultant/advisory board member for Genentech. A.E. Oro reports receiving a payment for non–exempt payment of page charges. This article must therefore be hereby marked to A.R. Segal.

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Authors’ Contributions
Conception and design: X. Zhao, T. Ponomaryov, R.A. Segal, F.B. Kellner, R.A. Segal. Development of methodology: X. Zhao, T. Ponomaryov, R.A. Segal. E. Pak. Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Zhao, T. Ponomaryov, R.A. Segal, F. Zhao. Analysis and interpretation of data (e.g., statistical analysis, biospecimens, computational analysis): X. Zhao, T. Ponomaryov, R.A. Segal, F. Zhao. Writing, review, and/or revision of the manuscript: X. Zhao, T. Ponomaryov, R.A. Segal, F. Zhao, E. Pak. Administrative, technical, or material support (e.g., reporting or organizing data, constructing databases): X. Zhao, R.A. Segal. Study supervision: X. Zhao, R.A. Segal.

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Consistent with our findings, ectopic expression of Eaz (embryonic stem-cell-enriched Ra), which is structurally similar to ERK (53), increases leptomeningeal metastasis in models of Shh subtype medulloblastoma, and these metastatic cells differ genetically and epigenetically from primary tumor cells (54, 55). Together, these data indicate that RAS activation results in resistance to Smo inhibitors and alter tumor characteristics.

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Clinical studies of resistant BCC also suggest a role of RAS/MEK signaling in tumor evolution. Several studies have reported occurrences of SCCs during treatment of BCC with vismodegib (19–26). Sequencing of matched pre- and posttreatment skin tumor samples supports the hypotheses that BCC tumor acquires RAS/ERK and/or PI3K resistance by which Shh pathway-dependent tumor cells evolve and escape Shh signaling dependence. Future studies are required to assess the prevalence of this resistance mechanism in patients.

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RAS Drives Drug Resistance and Tumor Evolution in Shh Tumors


Appendix III: Coordinate activation of Shh and PI3K signaling in PTEN-deficient glioblastoma: new therapeutic opportunities
Coordinate activation of Shh and PI3K signaling in PTEN-deficient glioblastoma: new therapeutic opportunities

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In glioblastoma, phosphatidylinositol 3-kinase (PI3K) signaling is frequently activated by loss of the tumor suppressor phosphatase and tensin homolog (PTEN)1. However, it is not known whether inhibiting PI3K represents a selective and effective approach for treatment. We interrogated large databases and found that sonic hedgehog (SHH) signaling is activated in PTEN-deficient glioblastomas. We demonstrate that the SHH and PI3K pathways synergize to promote tumor growth and viability in human PTEN-deficient glioblastomas. A combination of PI3K and SHH signaling inhibitors not only suppressed the activation of both pathways but also abrogated S6 kinase (S6K) signaling. Accordingly, targeting both pathways simultaneously resulted in mitotic catastrophe and tumor apoptosis and markedly reduced the growth of PTEN-deficient glioblastomas in vitro and in vivo. The drugs tested here appear to be safe in humans; therefore, this combination may provide a new targeted treatment for glioblastoma.

Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor. Currently, the average time from diagnosis to death is 15–18 months1, and therefore new treatment approaches are needed. 36% of GBMs harbor loss or inactivation of PTEN1, a lipid phosphatase that is a key negative regulator of PI3K signaling. However, it is unclear whether tumors deficient for PTEN are susceptible to PI3K inhibitors4,5.

Here we describe a therapeutic approach that targets PTEN-deficient GBMs. In two large data sets, PTEN mRNA expression varies more than tenfold among GBMs3. We divided a data set of 100 GBMs in two groups: GBMs that express low levels of PTEN and all other tumors (Supplementary Fig. 1a). In both data sets, GBMs with decreased PTEN expression exhibited increased SHH pathway activation, as assessed by expression of GLI1 and GLI2, which encode transcription factors that are critical for SHH signaling (Fig. 1a and Supplementary Fig. 1b). Moreover, PTEN expression negatively correlated with the combined mRNA expression of GLI1 and GLI2 across each data set (Spearman correlation coefficient, −0.23 and P = 0.02 for the data set of 100 GBMs and Spearman correlation coefficient, −0.23226 and P = 2 × 10−4 for the TCGA data set). Further analysis of the TCGA data demonstrated that GLI2 expression was higher in tumors with PTEN copy loss (Fig. 1b and Supplementary Fig. 1c), and PTEN expression correlated with PTEN copy number. These data indicate that PTEN-deficient GBMs exhibit activation of SHH signaling in addition to the known activation of PI3K cascades.

We tested the effects of selective PI3K and SHH inhibitors on neurosphere cultures from a human PTEN-deficient GBM (HbT70) and a PTEN-expressing GBM (HhT75a) (Fig. 1d). Neurosphere cultures recapitulate critical features of primary tumors, including somatic mutations, antigenic properties, tumor initiation and angiogenic activity7. Treatment with the PI3K inhibitor NVP-BKM120 (ref. 4) at 100 nM (half-maximum inhibitory concentrations (IC50) of 52, 166 and 116 nM for p110α (PIK3CA), p110β (PIK3CB) and p110γ (PIK3CG), respectively)3 did not alter the cell viability of PTEN-deficient or PTEN-expressing neurospheres (Fig. 1c,d). We saw no effect on viability when we treated the neurospheres with NVP-LDE225, an inhibitor of smoothened (SMO), which prevents SHH signaling8. In contrast, in PTEN-deficient GBM neurosphere cultures only, we found a significant reduction in viability with a combination

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We analyzed responses to combination therapy in monolayer cultures of hBT70 and two additional PTEN-deficient tumors (hBT112 and hBT145) (Fig. 1c)12. Combination therapy led to an efficacious and synergistic reduction in viability in all of the PTEN-deficient tumors tested (Fig. 1f) (two-way analysis of variance (ANOVA) factorial interaction, $F = 10.27, DF_n = 1, DF_d = 8, P = 0.0125$) but did not alter the growth of PTEN-expressing GBMs (Fig. 1c,g). As human GBMs vary in several additional characteristics (Supplementary Fig. 1b), we asked whether PTEN expression is a critical determinant of viability response. Two different shRNAs against PTEN introduced

**Figure 1** Coordinate activation of the SHH and PI3K pathways in PTEN-deficient glioblastoma. (a) Expression of GLI1 and GLI2 in PTEN-deficient and PTEN-expressing GBMs. Shown are robust z-scores for GLI1 and GLI2 (Lawrence Berkeley National Laboratory (LBNL) database, 40% cutoff). $P < 0.015$. **$P = 1.5 \times 10^{-5}$.** (b) GLI2 expression and PTEN copy number (Broad Institute database $P = 0.0002$ (Supplementary Fig. 1b). (c) PTEN immunoblot in GBMs, with actin used as the loading control. (d) hBT70 and hBT75 GBM neurospheres after treatment with vehicle, NVP-LDE225 (LDE225; 1 μM), NVP-BKM120 (BKM120, 100 nM) or a combination (combo) of the two drugs. Scale bar, 1 mm. (e) Quantification of the viability of the hBT70 tumors in d. $n = 3$ independent experiments. Two-way analysis of variance (ANOVA) factorial interaction was used to identify synergy in e-h and j. $F = 5.19, DF_n = 1, DF_d = 8, P = 0.0523$ compared to vehicle. *$F = 5.98, DF_n = 1, DF_d = 8, P = 0.0403$. (f) Viability of PTEN-deficient lines (hBT70, hBT112 and hBT145) after treatment with the indicated therapies. $n = 3$ independent experiments. *$F = 10.27, DF_n = 1, DF_d = 8, P = 0.0125$. (g) Viability of PTEN-expressing lines (hBT75, hBT188 and hBT239) after treatment with the indicated therapies. $n = 3$ independent experiments. $F = 0.09, DF_n = 1, DF_d = 8, P = 0.7749$ (NS, not significant). (h) PTEN immunoblot in GBM cells, with actin used as the loading control. shControl, nonspecific control shRNA; shPTEN1, one shRNA specific to PTEN; shPTEN2, a second shRNA specific to PTEN. (i) PTEN, GLI1 and GLI2 mRNA levels normalized to those of GAPDH, with results for each treatment normalized to those of the group not treated with virus. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, $^1P = 0.06$. $n = 8$ independent experiments for hBT188 in the top graph. For shControl, $P = 0.36$ for PTEN, $P = 0.72$ for GLI1, and $P = 0.20$ for GLI2; for shPTEN1, $P = 0.03$ for PTEN, $P = 1.1 \times 10^{-12}$ for GLI1, and $P = 0.0004$ for GLI2; for shPTEN2, $P = 0.03$ for PTEN, $P = 0.0003$ for GLI1, and $P = 9 \times 10^{-06}$ for GLI2. $n = 6$ independent experiments for hBT239 cells in the bottom graph. For shControl, $P = 1.3$ for PTEN, $P = 1.1$ for GLI1, and $P = 2.72$ for GLI2; for shPTEN1, $P = 0.03$ for PTEN, $P = 0.0002$ for GLI1, and $P = 0.0008$ for GLI2; for shPTEN2, $P = 0.03$ for PTEN, $P = 4.3 \times 10^{-14}$ for GLI1, and $P = 0.06$ for GLI2. Statistical analyses in i were performed using $t$-test with Bonferroni post test. (j) GBM responses to therapies after PTEN knockdown in hBT188 cells ($n = 10$ replicates, with two technical replicates in each 5×5 experiments; shPTEN1: *$F = 4.69, DF_n = 6, DF_d = 81, P = 0.004$; shPTEN2: *$F = 3.10, DF_n = 6, DF_d = 81, P = 0.008$) or hBT239 cells ($n = 9$ replicates with three technical replicates in each 5×5 experiments; shPTEN1: *$F = 2.59, DF_n = 6, DF_d = 72, P = 0.02$; shPTEN2: *$F = 2.47, DF_n = 6, DF_d = 72, P = 0.03$). All error bars throughout the figure represent the s.e.m.
LETTERS

Figure 2 A combination of PI3K and SMO inhibition reduces tumor growth in vivo. (a) Relative bioluminescence during 80 d of treatment of intracranial luciferase-expressing hBT112 tumors (n = 10 mice per group). Vehicle, NVP-LDE225 (60 mg per kg body weight), NVP-BKM120 (30 mg per kg body weight) or combination therapy was given daily. (b) Relative bioluminescence during 100 d of treatment in mice implanted with luciferase-expressing hBT1145 cells (n = 10 mice per group). The same drugs were given as in (a) but in cycles of 3 weeks on and 1 week off. Animals with no tumors and nontumor deaths were censored (<1 animal per group). The treatment groups differed by two-way ANOVA (P < 0.0001), and the combination treatment differed from NVP-BKM120 treatment after Bonferroni post test (P < 0.0001). (c) Kaplan-Meier analysis showing increased survival with combination therapy or NVP-BKM120 (P < 0.0001) by two-way ANOVA with Bonferroni post test. (d) Relative bioluminescence of mice treated with the combination therapy or NVP-BKM120 on days 145 (n = 7 mice for NVP-BKM120 and combination) and 162 (n = 4 mice for NVP-BKM120, n = 6 mice for combination). *P = 0.03, **P = 0.01 by t test. (e) Three-dimensional reconstructions of MRI scans taken on day 65 (vehicle and NVP-LDE225) or day 77 (NVP-BKM120 and combination treatment). Blue indicates tumor tissue. The arrowhead points in the anterior direction, and the tumor volumes are indicated at the bottom of each image. (f) Immunostaining for human NUMA showing tumor cells within mouse brains (at day 65 for vehicle and NVP-LDE225 and at day 80 for NVP-BKM120 and combination treatment). Scale bar, 5 mm. Error bars throughout the figure represent the s.e.m.

in two distinct PTEN-expressing human GBMs decreased PTEN expression by 50–70% (Fig. 1b). Acute reduction of PTEN expression increased the expression of GLI1 and GLI2 (Fig. 1i), whereas acute overexpression of PTEN decreased the expression of these SHH pathway components (Supplementary Fig. 1f). Notably, when PTEN expression was stably reduced in two distinct GBM tumors, combination therapy with NVP-BKM120 and NVP-LDE225 decreased the viability of previously resistant cells (Fig. 1j). Thus, PTEN expression is not merely correlative for response but has a causative role as well.

On the basis of these promising results, we analyzed PTEN-deficient tumors in vivo using an orthotopic xenograft model13. We intracranially injected 1 × 10⁶ human GBM cells (hBT112) expressing luciferase into nu/nu mice and then monitored tumor size with bioluminescence. We obtained similar results in a second tumor tested (Fig. 2d). Thus, PTEN-deficient human GBM cells (hBT112) expressing luciferase into nu/nu mice and then monitored tumor size with bioluminescence. We initiated treatment only after tumors showed exponential growth. Mice treated with vehicle or NVP-LDE225 exhibited rapid increases in bioluminescence (Fig. 2a). NVP-BKM120 treatment initially slowed tumor growth, but this effect was transient. In contrast, mice treated with combination therapy showed stable bioluminescence throughout the experiment, indicating substantially reduced tumor growth (P = 0.026 compared to vehicle treatment) (Fig. 2a).

We obtained similar results in a second tumor tested in vivo (hBT145) (Fig. 2b). Moreover, whereas the groups treated with NVP-BKM120 or the combination therapy showed improved survival compared to the vehicle-treated and NVP-LDE225-treated groups (Fig. 2c), the tumor burden in animals that survived more than 4 months was reduced in mice treated with the combination therapy (Fig. 2d).

Consistent with the results from the bioluminescence studies, magnetic resonance imaging (MRI) analyses and histological examinations showed that combination therapy diminished tumor sizes (Fig. 2e,f) and decreased the dissemination of tumor cells (Fig. 2f), as assessed by staining for human nuclear mitotic apparatus protein (NUMA).

To examine the cellular basis for the synergistic effects of the combination treatment, we labeled glioblastoma cells with DiI (1,1'-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and imaged individual cells over time (Fig. 3a). Combination therapy decreased proliferation and increased cell death (Fig. 3b). Accordingly, combination therapy increased apoptosis both in vitro, as demonstrated by activated caspase-3 (Fig. 3c,d and Supplementary Fig. 2a), and in vivo, as demonstrated by TUNEL staining of GBM xenografts (Fig. 3e,f). Combination therapy also affected tumor cell...
Combinational therapy clearly achieves targeted responses: NVP-BKM120 or NVP-LDE225 alone reduced the amounts of phosphor-S6 (pS6) and tumor cell viability (Fig. 4a and Supplementary Fig. 3a). Similarly, in vivo, both NVP-LDE225 and the combination treatment reduced the amounts of pS6 (Fig. 4b). Stimulation of GBM cells with SAG, a SMO agonist, caused dose-dependent increases in the amounts of pS6, implicating the SHH pathway in S6 activation (Fig. 4b). Furthermore, a small-molecule inhibitor of S6K (PP-4708671)* led to dose-dependent decreases in the amounts of pS6 and tumor cell viability (Fig. 4c and Supplementary Fig. 3d), suggesting that S6K is a critical interaction node for the combination therapy. Combination therapy also synergistically decreased expression of cyclin D1 (CCND1), which is regulated by the PI3K, SHH
and S6K pathway26–28 (Supplementary Fig. 3e). Thus, although NVP-BKM120 and NVP-LDE225 each successfully attack appropriate targets in Pten-deficient GBM cells, both P38 and SHH signaling must be targeted to maximally diminish S6K activity and repress tumor growth.

To identify additional consequences of the combination therapy on a genome-wide scale, we used Affymetrix microarrays with two different Pten-deficient GBMs treated with either single drugs or the combination therapy. Genes markedly affected by the combination therapy in hBT70 tumors, hBT112 tumors or both (Supplementary Fig. 3f and Supplementary Table 1) included several genes that are implicated in GBM prognosis or have been identified as targets of the SHH, P38, or S6K pathways22–24 (Fig. 4). The studies presented here indicate that SHH signaling and P38 pathways are both activated in PTEN-deficient GBMs, and therapies that target only P38 or P38 have limited efficacy in these tumors23. Instead, a combination of P38 and SHH signaling inhibitors successfully targets both pathways and achieves a synergistic effect on S6K signaling. Combination therapy causes apoptosis as well as mitotic catastrophe and substantially reduces tumor growth in vitro and in vivo.

Activation of SHH signaling has been reported previously in GBMs26–28. Here we found that expression of GLI transcription factors correlates with both PTEN mRNA levels and PTEN copy number in large GBM databases. Our data indicate a causal connection between PTEN and SHH signaling and identify S6K signaling as a critical node of interaction28–31 (Fig. 4). In the absence of PTEN, decreased degradation of PIP3 lipids results in activation of AKT, mTOR, and S6K signaling. S6K activity in turn enhances GLI-dependent transcription29,32–34. Stimulation of the SHH receptor, SMO, further enhances S6K signaling. Accordingly, PTEN deficiency increases P38, SHH, and S6K signaling, and thus a combination of P38 and SHH inhibitors results in apoptotic death of PTEN-deficient GBM cells.
Previous studies have suggested that high doses of inhibitors of either the SHH or PI3K pathway reduce GBM neurosphere growth, colony formation or both.25–28 Here we show efficacy using doses of the PI3K inhibitor NVP-BKM120 and the SHH pathway inhibitor VOP-LDE225 that are achievable in vivo through oral administration. These drugs cross the blood-brain barrier, have acceptable toxicity profiles and have now entered clinical trials.29 (NCT01576666 at clinicaltrials.gov). Our results indicate the need to monitor PTEN status and p56K activation in clinical studies and highlight the importance of testing these agents as a combination therapy for glioblastoma.10,29 (NCT01576666 at clinicaltrials.gov).

METHODS

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

Access codes

The microarray data shown in Supplementary Figure S3 have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE49416.

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

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AUTHOR CONTRIBUTIONS

M.G.F., S.K.D., E.P. and R.A.S. wrote the manuscript with input from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Genomic analysis. The GEO database was used to examine PTEN, GLI1 and GLI2 in 100 high-grade gliomas (H. Phillips, GEO Data Set GDS18193). The mean robust z-scores for GLI1 and GLI2 were determined for the set of tumors with lower PTEN expression (20–40% of tumors), as well as for the rest of the tumors with higher PTEN expression. Student’s t test was used to determine whether the z-scores for GLI1 and GLI2 in the two groups were significant. The data shown in Supplementary Figure 1a used a cutoff of 22% (Supplementary Fig. 1b), this difference was also significant (P < 0.05) for GLI1 and GLI2 using a cutoff of 34% or 39%. A similar analysis was carried out for the TCGA data (LBl) shown in Figure 1a. PTEN copy number from TCGA was categorized as either a copy loss (log, ratio < 0.3) or copy neutral. The −0.3 threshold represents a minimum in copy number distribution (Supplementary Fig. 1b).

GBMs. All work with human subjects was reviewed by the Institutional Review Board Committees of the Brigham and Women’s Hospital and the Dana-Farber Cancer Institute for appropriate use and ensure that that informed consent was obtained from all subjects when required and appropriate waiver-of-consent requirements were obtained for minimal risk studies.

Animal studies. All experimental procedures were done in accordance with the US National Institutes of Health guidelines and were approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee.

GBMs in vitro. Human GBM tumor-initiating cell lines (hBT112, hBT145, hBT188 and hBT239) were derived from patients treated at Brigham and Women’s Health according to Institutional Review Board–approved protocols. hBT70 and hBT75 lines were from C. David James (University of California San Francisco). Cells from glioblastoma biopsies were implanted into nude mice. Dissected xenografts were processed as described. For adherent conditions, cells were cultured as described. All cultures were grown in NS-A stem cell medium (Stem Cell Technologies) with epidermal growth factor (EGF) (20 ng ml−1), basic fibroblast growth factor (bFGF) (20 ng ml−1) and 0.2% heparin (1 μg ml−1). Viability was determined by Trypan Blue exclusion or a CellTiter-Glo Luminescent Cell Viability Assay (Promega) at the end of treatment (day 7 for monolayer assays and day 14 for neurosphere assays). PTEN knockdown was achieved in hBT188 and hBT239 cultures after 48 h of infection with a lentivirus expressing one of two distinct shRNAs to PTEN. Cells were used after 7 d of puromycin selection (1 μg ml−1) in assays of viability, immunoblotting and real-time RT-PCR. PTEN overexpression was achieved in hBT112 cultures after 48 h of infection with a retrovirus expressing full-length PTEN. Cells were used after 1 d of puromycin selection (1 μg ml−1) in assays of real-time RT-PCR.

Live imaging. Human GBM tumor-initiating cells (hBT70) were stained with DiI (Vybrant Multicolor Cell-labeling Kit, Invitrogen) for 20 min at 37 °C, mixed with unlabeled hBT70 cells and plated on laminin-coated (10 μg ml−1 Invitrogen) 12-well plates. 24 h after plating, we added drugs or vehicle as indicated. Dishes were placed within an incubation chamber fitted around a 37 °C microscope, an Orca ER camera and Andor iQ 2.3 software.

In vivo experiments. For orthotopic transplants, hBT112 or hBT145 neurospheres expressing luciferase were dissociated and resuspended in HBSS at 1 × 106 cells ml−1. 1 μl was injected stereotactically (coordinates: x = −2, y = 0, z = −2) into nu/nu mouse brains. After 6 weeks, animals were imaged weekly. Animals with increasing bioluminescence imaging were randomly assigned to four groups. Drugs were administered by oral gavage once daily; ten mice received vehicle control, ten mice received NVP-LDE225 at 60 mg per kg body weight, ten mice received NVP-BKM120 at 30 mg per kg body weight, and ten mice received the combination of NVP-LDE225 and NVP-BKM120 with 30 min between the dosings of the two drugs. MRI scans were performed on two animals per group at the end of treatment on day 65 (vehicle and NVP-LDE225) or day 77 (NVP-BKM120 and the combination treatment).

T2-weighted images were acquired, three dimensional–rendered models were generated, and tumor volume was measured using 3D Slicer with a thresholding method. Animals were euthanized if they became ill.

Inhibitors. NVP-LDE225, NVP-BKM120, NVP-LEQ506 and NVP-RAD001 were from Novartis. NVP-LDE225 and NVP-BKM120 were formulated as diphasate salt in 0.5% methylcellulose and 0.5% Tween 80 (Fisher) for the in vivo studies. For in vivo studies, NVP-LDE225, NVP-BKM120, NVP-LEQ506 and NVP-RAD001 were suspended in DMSO and used at the concentrations indicated. GDC-0941 was from Sai Advantum Pharma and ShangHai Biochempartner. PF-4708671 was a generous gift from N. Gray. Cyclopamine was from LC Laboratories.

shRNAs for PTEN. hBT188 and hBT239 neurospheres were cultured as a monolayer on laminin-coated dishes. The lentivirus (shControl) used targeted luciferase. The targeting sequence for shPTEN is CCACACGCTTAGAA CTTATCAA, and the targeting sequence for shPTEN2 is AGCCGCTATGTGATTATTAT. Protamine was included during lentiviral infection; after infection, cells were selected in puromycin 1 μg ml−1 for 1 week.

Immunoblotting. GBM tumor-initiating cells were lysed in modified RIPA buffer (30 mM Na–Tris, pH 7.4, 150 mM NaCl, 1% (vol/vol) NP-40, 0.25% sodium deoxycholate, 1 mM dithiothreitol, 10 mM NaF, 1 mM active sodium vanadate, 1 mM PMFS and 1× proteinase inhibitor). Lysates were separated by 4–12% SDS-PAGE. The antibodies used were as follows: rabbit antibody to PTEN (1:1,000), rabbit antibody to AKT (1:1,000), rabbit antibody to pAKT (Ser473) (1:2,000), rabbit antibody to pJAK (Thr508) (1:1,000), rabbit antibody to cyclin D1 (1:5,000), rabbit antibody to p70 S6K (1:1,000), rabbit antibody to p70 S6K (1:1,000), mouse antibody to PTEN (1:1,000), and rabbit antibody to actin (1:1,000). The antibody to cyclin D1 was from Millipore, all other antibodies were from Cell Signaling Technologies. Bands were visualized with secondary antibodies conjugated to horseradish peroxidase and detected by chemiluminescence with an ImmunoStar kit (Amersham), and band intensity was quantified with ImageJ 1.42q and was normalized to actin.

Immunocytochemistry. hBT70 cells were plated on laminin-coated coverslips and treated as indicated for 7 d. Cells were fixed for 15 min in 4% paraformaldehyde and blocked in 10% normal goat serum, 1% BSA and 0.1% Triton X-100 in PBS for 30 min at room temperature before staining for activated caspase-3 or were fixed at −20 °C for 10 min in precooled 100% methanol, permeabilized at room temperature for 5 min in 0.2% Triton X-100 and blocked at room temperature for 30 min in 5% BSA and 0.1% Triton X-100 for spindle staining. Samples were incubated with primary antibodies for 2 h at room temperature and then with antibody to rabbit Alexa Fluor 488 (Invitrogen, 1:800) or antibody to mouse Alexa Fluor 546 (Invitrogen, 1:800) and DAPI for 1.5 h at room temperature. All coverslips were mounted using ProLong Gold Antifade Reagent (Invitrogen). The primary antibodies used were as follows: rabbit antibody to activated caspase-3 (Abcam, ab83847, 1:1,000), rabbit antibody to β-tubulin (Sigma Aldrich, T5192, 1:400) and mouse antibody to acetylated tubulin (Sigma Aldrich, #4793, 1:800).

Images were obtained with a Nikon E800 microscope, a CoolSnap EZ camera and ND Imaging software using a 40× oil objective for β-tubulin and acetylated tubulin and a 20× objective for activated caspase-3. Images of spindles were acquired using a 100× objective, a Yokogawa Spinning Disk Confocal microscope, an Orca ER camera and Andor iQ 2.3 software.

Immunohistochemistry of in vivo tumors. Specimens were fixed in 10% buffered formalin and embedded in paraffin. 5-μm sections were stained with H&E or used for immunohistochemical studies. After antigen retrieval (citrate, high temperature) antibodies to NUMA (Epitomics, S2825, 1:200) and pS6 (Cell Signaling, #2211S, 1:200) were used and visualized using the Envision Plus Detection System (Dako, Carpinteria, CA). Neuropathologists performed all evaluations and interpretations of brain sections (K.L.L. and S.R.). TUNEL staining was performed using the DeadEnd Fluorometric TUNEL System (Promega).
Real-time RT-PCR. GBM tumor-initiating cells were collected and lysed in either RNAlater (Life Technologies) or Buffer RLT (Qiagen), and RNA was isolated with the RNeasy Plus Mini kit (Qiagen). Reverse transcription was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time RT-PCR was performed using TaqMan Gene expression assays for human PTEN (Hs_00181117_m1), human GLI1 (Hs00171790_m1), human GLI2 (Hs_00257977_m1), human PTCH1 (Hs00181117_m1) and human GAPDH (TaqMan Pre-developed Assay reagents). Each analysis was performed in triplicate, and the results were normalized to GAPDH for each sample.

Microarray analysis. RNA was isolated as described from hBT112 and hBT70 cells after 5 d of treatment and then applied to Affymetrix Human Genome U133A 2.0 Arrays at the Dana-Farber Cancer Institute microarray facility (http://macf-web.dcli.harvard.edu/). Biological duplicate samples were tested, and arrays were visualized and analyzed using dChip software (http://macf-web.dcli.harvard.edu/).

Statistical analyses. All analyses were done using Microsoft Excel or Prism GraphPad 5.00 for Mac OS (San Diego California, www.graphpad.com), except for the analysis of tumor growth in vivo, which was done using SAS 9.2 (SAS Institute Inc., Cary, NC).

The synergistic effects of protein, mRNA levels or viability were analyzed by a two-way ANOVA factorial design with Bonferroni post test. Tests of efficacy were done using one-way ANOVA with Bonferroni post test, Student’s two-tailed, type 2 test or t-test, as indicated.

To combine multiple experiments, results in each experiment were normalized to those of the associated vehicle control before calculating the mean and s.e.m. For hBT112 or vivo experiments, normality was checked for all measurements using the Kolmogorov-Smirnov test, and analysis of covariance tests were performed to explore treatment group effects as well as essential covariates among baseline weight, baseline log bioluminescence and relative percent change of weight. In longitudinal modeling, five forms of linear mixed models were considered: (i) linear mixed model with random intercept, (ii) individual linear mixed model with random intercept, (iii) random regression model, (iv) random regression model in which the random slope and intercept were assumed to be independent and (v) linear mixed model in which a within-subject covariance-variance structure was specified. Under the respective models, a group effect (categorized as the vehicle control treatment or treatment with NVP-BKM120, NVP-LDE225 or the combination of NVP-BKM120 and NVP-LDE225) was introduced as an independent variable, and we examined different combinations of the covariates and variance-covariance specifications (unstructured, 1-autoregressive and compound symmetry). Among all the fit models, the best model was selected on the basis of the Akaike information criterion (AIC) and the significance of the covariates. In order to make an inference regarding the group effect, contrast tests were performed using the selected best model.

This study had an exploratory nature and there was no prespecified effect size. The sample size was chosen based on the study feasibility and not statistical power. Ten mice per group or a total of 40 mice were used in the bioluminescence study. The study was longitudinal, generating data at 11 follow-up time points per mouse. Post hoc power calculation is generally considered to be invalid, so we do not provide a statistical justification based on power. However, we observed a total of 440 follow-up samples, which is considered to be adequate to allow us to perform a longitudinal analysis. The sample size was chosen to enable a 30% difference to be detected with 80% power in a two-sided test of significance. Animals without evidence of tumor by imaging were not entered onto treatment. Animals were assigned to treatment groups to achieve cohorts with statistically similar mean bioluminescence levels. The studies were unblinded.
REFERENCES


