Hedgehog signaling drives medulloblastoma growth via CDK6

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Introduction
Medulloblastoma, an aggressive cancer of the cerebellum, is among the most common pediatric brain tumors (1). Transcriptional profiling studies reveal that medulloblastomas exist as 4 main molecular subgroups (2). Approximately one-third of medulloblastomas are associated with misactivation of the Hedgehog (Hh) pathway, a signal transduction pathway that is essential for development (3). Vertebrate Hh signals are transduced through the primary cilium, an antenna that projects from the surface of most cells. Cells of the cerebellar external granule layer (EGL) give rise to Hh-associated medulloblastoma and are ciliated (4, 5). Other Hh-related cancer cells, such as basal cell carcinoma cells, are also ciliated, and disrupting either cilia or ciliary Hh signaling blocks cancer growth in both basal cell carcinoma and medulloblastoma (5, 6).

Hh ligands relieve Patched1 (Ptc1) repression of Smo1 (SMO), allowing SMO to localize to cilia and activate GLI family zinc finger 2 (GLI2), the principle effector of the Hh transcriptional program (5). The targets of GLI2 that drive uncontrolled cell proliferation in cancer are poorly understood. Here, we demonstrate that GLI2 binds to the Cdk6 promoter to induce cell proliferation. Inhibiting CDK6 blocks the growth of Hh-associated medulloblastoma in vivo, suggesting that pharmacologic inhibition of CDK6 may be an effective strategy for patients with Hh-associated cancers.

Results and Discussion
To study how misactivation of GLI2 causes cancer, we used the Floxin system to generate a Gli2-knockin allele that encodes a fusion of GLI2 to EGFP and FLAG tags (Gli2-EGFP) (7). Mice homozygous for the Gli2-EGFP allele are viable and morphologically indistinguishable from WT, revealing that this fusion protein is functional (8). To investigate the function of GLI2 in medulloblastoma, we incorporated the Gli2-EGFP allele into 2 different genetic mouse models of Hh-associated medulloblastoma. Hh signaling induced GLI2 binding to the Cdk6 promoter and activated Cdk6 expression, thereby promoting uncontrolled cell proliferation. Genetic or pharmacological inhibition of CDK6 in mice repressed the growth of Hh-associated medulloblastoma and prolonged survival through inhibition of cell proliferation. In human medulloblastoma, misactivation of Hh signaling was associated with high levels of CDK6, pointing to CDK6 as a direct transcriptional target of the Hh pathway. These results suggest that CDK6 antagonists may be a promising therapeutic approach for Hh-associated medulloblastoma in humans.
of uncontrolled cell proliferation in medulloblastoma regardless of genetic etiology (Supplemental Figure 1E). In support of this hypothesis, small molecule inhibition of CDK6 confers a survival benefit in mice bearing patient-derived xenographs of group 3 medulloblastomas (14).

The levels of the CDK6-interacting cyclin cyclin D1 were also elevated in Hh pathway–associated medulloblastoma (Figure 1C, Supplemental Table 1, and Supplemental Figure 2A). We therefore assessed phosphorylated RB levels and found them to be dramatically increased in Hh pathway–associated medulloblastoma (Figure 1C). Consistently, medulloblastomas displayed markedly elevated expression of E2F target genes (Supplemental Figure 2A), further suggesting that misactivation of Hh signaling may drive cell cycle progression via CDK6 (15). Of note, the read count of the related mitogenic kinase Cdk4 was higher than that of Cdk6 in Hh-associated medulloblastoma, but the differential expression of Cdk6 at the promoter that is involved in Hh signaling–mediated limb development (site 4, Figure 2, B and C, and Supplemental Figure 3, A–F) (11). Consistent with Hh pathway–associated binding of a transcriptional activator, site 4 also exhibited an enrichment of H3K27 acetylation, which is associated with transcriptional activation, and a suppression of H3K27me3, which is associated with transcriptional repression, in medulloblastoma (Figure 2D and Supplemental Figure 3G). Similarly, ChIP of NIH/3T3 cells expressing EGFP-GLI2 showed increased occupancy of EGFP-GLI2 at site 4 upon activation of Hh signaling (Figure 2E and Supplemental Figure 3, H–J).

To test whether GLI2 binding to the Cdk6 promoter is sufficient to activate transcription, we made luciferase reporter constructs containing truncated Cdk6 promoter sequences. Transfection of reporters into NIH/3T3 cells revealed that Cdk6 promoter sequences that included site 4 were sufficient to confer responsiveness to SAG-mediated activation of the Hh pathway (Figure 2F). Similarly, Cdk6 promoter sequences that included site 4 conferred responsiveness to a constitutively active form of GLI2, GLI2-CLEG (Figure 2G). In contrast, a Cdk6 promoter sequence that did not include site 4 was not responsive to either SAG or GLI2-CLEG (Figure 2, F and G). Further-
removed CDK6 from the important for Hh-associated cancer growth, we genetically tal Figure 4, A and B) (18). To test whether CDK6 is functionally SmoM2c removing CDK6 function prolonged median survival in Math1-Cre 3B and Supplemental Figure 4, C and D). Moreover, genetically loblastoma and partially restored cerebellar architecture (Figure 3D and Supplemental Figure 4, C and D). Homozygous genetic deletion of Cdk6 3A). Homozygous genetic deletion of Cdk6 allele (135 versus 68 days, Figure 3D). Pharmacologic inhibition of CDK4/6, palbociclib. A positive control, the SMO inhibitor vismodegib, reduced medulloblastoma weight (32% ± 2%, Figure 3A) (19). Pharmacologic inhibition of CDK4/6 with palbociclib reduced tumor weight in both Math1-Cre SmoM2α (31% ± 3%, Figure 3, A and E) and Math1-Cre Ptc1-1 (41% ± 5%, Figure 3F) mice to an extent similar to that seen with vismodegib. Much like genetic deletion of Cdk6, palbociclib also reduced the size of tumors, decreased the prevalence of small round blue cells, partially restored cerebellar architecture, and prolonged survival (Figure 3D and Supplemental Figure 4, C and D).

To confirm the efficacy of CDK6 inhibition for medulloblastoma, we treated Math1-Cre Ptc1-1 mice with a different small molecule CDK4/6 antagonist abemaciclib, which also reduced the size of tumors (14% ± 2%, Figure 3F). As both palbociclib and abemaciclib inhibit CDK4 in addition to CDK6, we treated Math1-Cre SmoM2α Cdk6α/α mice with palbociclib to test whether inhibition of CDK4 contributes to their effect on medulloblastoma size. We did not detect a difference in Math1-Cre SmoM2α Cdk6α/α tumor weight with and without palbociclib, suggesting that CDK4 is not a significant driver of Hh-associated medulloblastoma growth (Figure 3A).

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more, multimerized site 4 without surrounding Cdk6 sequences was sufficient to confer transcriptional responsiveness to GLI2-CLEG (Figure 2H and Supplemental Table 2).

Cdk6-null mice have no overt developmental phenotypes, and Cdk6 is not required for cerebellar development (Supplemental Figure 4, A and B) (18). To test whether CDK6 is functionally important for Hh-associated cancer growth, we genetically removed CDK6 from the Math1-Cre SmoM2α medulloblastoma model. Homozygous genetic deletion of Cdk6 (Math1-Cre SmoM2α Cdk6α/α) reduced the weight (32% ± 2%) and size of tumors relative to those with either 1 or 2 copies of the Cdk6 allele (Figure 3A). Homozygous genetic deletion of Cdk6 also reduced the prevalence of small round blue cells that are characteristic of medulloblastoma and partially restored cerebellar architecture (Figure 3B and Supplemental Figure 4, C and D). Moreover, genetically removing CDK6 function prolonged median survival in Math1-Cre SmoM2α animals (97 days versus 52 days) (Figure 3C). To confirm the involvement of CDK6 in Hh-associated medulloblastoma, we genetically removed CDK6 in a second tumor model, one that relies on the loss of the negative regulator of the pathway, PTCH1, rather than activation of SMO. As with the SMO misactivation tumors, homozygous genetic deletion of Cdk6 in the Math1-Cre Ptc1-1 tumors prolonged median survival relative to animals with 2 copies of the Cdk6 allele (135 versus 68 days, Figure 3D).

We hypothesized that, like genetic deletion of Cdk6, pharmacological inhibition of CDK6 would inhibit the growth of medulloblastoma. To test this hypothesis, we treated Math1-Cre SmoM2α and Math1-Cre Ptc1-1 mice with a small molecule inhibitor of CDK4/6, palbociclib. A positive control, the SMO inhibitor vismodegib, reduced medulloblastoma weight (32% ± 2%, Figure 3A) (19). Pharmacologic inhibition of CDK4/6 with palbociclib reduced tumor weight in both Math1-Cre SmoM2α (31% ± 3%, Figure 3, A and E) and Math1-Cre Ptc1-1 (41% ± 5%, Figure 3F) mice to an extent similar to that seen with vismodegib. Much like genetic deletion of Cdk6, palbociclib also reduced the size of tumors, decreased the prevalence of small round blue cells, partially restored cerebellar architecture, and prolonged survival (Figure 3D and Supplemental Figure 4, C and D).

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gest that simultaneous molecular inhibition of SMO and CDK6 may be an effective strategy for inhibiting the growth of Hh-associated medulloblastoma.

To understand the mechanism by which CDK4/6 inhibition attenuates the growth of Hh-associated medulloblastoma, we quantified tumor cell apoptosis and proliferation after palbociclib treatment in Math1-Cre SmoM2 cmice. Pharmacologic inhibition of CDK6 had no effect on tumor apoptosis (Supplemental Figure 4, F and G). In contrast, palbociclib reduced the amount of BrdU-positive cells by 35% ± 2%, indicating that CDK4/6 inhibition diminished cell proliferation (Figure 3G and Supplemental Figure 5A). As inhibiting CDK6 induces G1 arrest and cellular senescence (22, 23), we hypothesized that the effect of CDK4/6 inhibition on Hh-associated medulloblas-

ment, therapeutic doses of vismodegib are associated with premature growth plate fusion, but palbociclib is not (Supplemental Figure 4E) (21). To determine whether combination molecular therapy is an effective strategy for medulloblastoma, we treated Math1-Cre SmoM2 mice with vismodegib and palbociclib. We identified substantial morbidity with full-dose combination molecular therapy (150 μg/g vismodegib and 100 μg/g palbociclib). Thus, we reduced the dose of each agent (75 μg/g vismodegib and 50 μg/g palbociclib). Low-dose monotherapy with either agent failed to reduce tumor weights as much as full-dose treatment (vismodegib, 24% ± 2%; palbociclib, 28% ± 3%; Figure 3A). However, low-dose combination therapy reduced tumor weight more than either agent and to an extent comparable to that of full-dose monotherapy (39% ± 1%). These data sug-

Figure 3. Inhibition of CDK6 attenuates Hh-associated medulloblastoma growth. (A) Cerebella weight normalized to total brain weight from P35 mice (SmoM2 [n = 12, white], Math1-Cre SmoM2 [n = 7, light blue], and Math1-Cre SmoM2 Cdk6+/+ [n = 19, dark blue]) and P35 mice treated with pharmacologic agents (Math1-Cre SmoM2 with vismodegib 75 μg/g [n = 15, black], vismodegib 100 μg/g [n = 14, black], palbociclib 50 μg/g [n = 11, green], palbociclib 100 μg/g [n = 12, green], or vismodegib 75 μg/g, and palbociclib 50 μg/g [n = 13, orange], and Math1-Cre SmoM2 Cdk6+/+ with palbociclib 100 μg/g [n = 6, blue]). P < 0.04, t test. (B) P35 sagittal midline cerebellar H&E light micrographs. Scale bar: 1 mm (representative of 3 experiments). (C) Kaplan-Meier curves of 20 Math1-Cre SmoM2 (gray), 24 Math1-Cre SmoM2 Cdk6+/+ (light blue), and 25 Math1-Cre SmoM2 Cdk6+/+ mice. P < 0.0001, log-rank test. (D) Kaplan-Meier curves of 13 Math1-Cre Ptc1+/+ Cdk6+/+ (gray), 10 Math1-Cre Ptc1+/+ Cdk6+/+ (blue), and 8 Math1-Cre Ptc1+/+ Cdk6−/− mice treated with 100 μg/g palbociclib from P21 until death (green). P < 0.0001, log-rank test. (E) P35 brain micrographs. Scale bar: 5 mm (representative of 3 experiments). (F) Cerebella weight normalized to total brain weight of P35 mice, including 12 Ptc1+/+ (white), 12 Math1-Cre Ptc1+/+ (gray), and Math1-Cre Ptc1+/+ treated with 75 μg/g abemaciclib (n = 8, light green) or 100 μg/g palbociclib (n = 11, green). (G) BrdU quantification of Math1-Cre SmoM2 medulloblastomas. P < 0.03, t test. n = 3. (H) qRT-PCR of DAOY, D283, and D341 cells. P < 0.05, t test. n = 3. (I) Ki-67 quantification in DAOY, D283, and D341 cells. *P < 0.05, t test. n = 6.
toma is mostly cytostatic. In support of this hypothesis, tumor cell proliferation recovered following palbociclib withdrawal (Supplemental Figure 5, B and C).

To test the generalizability of CDK4/6 inhibition for other medulloblastoma molecular subgroups, we treated diverse human medulloblastoma cell lines with palbociclib and quantified cell proliferation. DA0Y medulloblastoma cells, representative of Hh-associated medulloblastoma, had elevated expression of Gli1, Ptc1, and Cdk6 relative to D283 and D341 medulloblastoma cell lines, which is representative of group 3 or group 4 medulloblastoma (Figure 3H) (24, 25). Consistently, palbociclib significantly reduced the amount of Ki-67–positive DA0Y cells in a dose-dependent manner and only mildly reduced Ki-67 expression in D283 and D341 cells (Figure 3I and Supplemental Figure 5D). These data suggest that CDK4/6 inhibition may be most effective in medulloblastoma tumors with elevated CDK6 expression.

In conclusion, we demonstrate that misactivation of Hh signaling in cancer induces CDK6 to drive medulloblastoma growth. The main transcriptional effector of Hh signaling, GLI2, binds to a site within the Cdk6 promoter to induce Cdk6. In turn, Cdk6 phosphorylates RB to activate E2F and induce medulloblastoma cell proliferation. Either genetic or pharmacologic inhibition of Cdk6 in 2 genetically distinct mouse models reduces medulloblastoma proliferation, reduces tumor burden, and prolongs survival. We propose that, as a direct transcriptional target of GLI2, Cdk6 is a principal means by which the Hh pathway activates the cell cycle in cancer. Therefore, we hypothesize that CDK4/6 inhibition will be an effective therapy for patients with Hh-associated medulloblastoma.

Methods
Please see Supplemental Methods for a detailed explanation of all experimental procedures.

Study approval. Animal experiments were conducted in a Laboratory Animal Resource Center per UCSF Institutional Animal Care and Use Committee–approved protocol AN098101.

Author contributions
DRR designed research studies, conducted experiments, acquired data, analyzed data, and wrote the manuscript. PKC conducted experiments, acquired data, and analyzed data. ALK conducted experiments, acquired data, and analyzed data. WM conducted experiments. NS provided reagents. JFR designed research studies and wrote the manuscript.

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