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Medulloblastoma, an aggressive cancer of the cerebellum, is among the most common pediatric brain tumors. Approximately one-third of medulloblastomas are associated with misactivation of the Hedgehog (Hh) pathway. GLI family zinc finger 2 (GLI2) coordinates the Hh transcriptional program; however, the GLI2 targets that promote cancer cell proliferation are unknown. Here, we incorporated a 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.

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Hedgehog signaling drives medulloblastoma growth via CDK6

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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 (PTCH1) repression of Smoothened (SMO), allowing SMO to localize to cilia and activate GLI family zinc finger 2 (GLI2), the principle effector of the Hh transcriptional program (3). 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 in response to Hh signals. 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.

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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 Cdk4 in Hh-associated medulloblastoma relative to normal cerebellum was 13.9-fold less than that of Cdk6 (Supplemental Figure 2, A and B, and Supplemental Table 1).

In human medulloblastoma, CDK6 expression was elevated even relative to that in highly proliferative neural progenitors (Supplemental Figure 2C), further raising the possibility that misactivation of Hh signaling induces super-physiological levels of CDK6 expression. To test whether Hh signals are sufficient to induce CDK6 expression, we activated NIH/3T3 cells, a Hh-responsive cell type, with SmoM2c Gli2-EGFP (16). Hh pathway activation in this setting induced elevation of diverse cell cycle effectors, including Ccnd1 and Cdk6 (Figure 1D). Concurrent inhibition of translation blocked the induction of Ccnd1 in response to Hh stimulation, but did not affect Cdk6 induction (Figure 2A). These data suggest that, whereas Hh signaling affects the expression of diverse cell cycle effectors, Cdk6 is exceptional in that it is a direct transcriptional target of the pathway.

The Cdk6 promoter binds GLI3, the principle repressor of the Hh transcriptional program, during limb-bud development (11, 17). ChIP analysis of GLI2-EGFP–binding sites in medulloblastomas from Math1-Cre SmoM2c Gli2-EGFP mice revealed that, compared with cerebella from control GLI2-EGFP–expressing mice, GLI2-EGFP is selectively enhanced at a previously identified cis-regulatory element at the Cdk6 promoter that is involved in Hh signaling–mediated limb development (site 4, Figure 2A). These data suggest that, whereas Hh signaling affects the expression of diverse cell cycle effectors, Cdk6 is exceptional in that it is a direct transcriptional target of the pathway.

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-
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 SmoM2 Cdk6−/− medulloblastoma model. Homozygous genetic deletion of Cdk6 (Math1-Cre SmoM2+ Cdk6−/−) 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 3A and E) and Math1-Cre Ptch1 c/c (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 Ptch1+/- 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 SmoM2+ (31% ± 3%, Figure 3, A and E) and Math1-Cre Ptch1+/- (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 Ptch1+/- with a small molecule inhibitor of 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 SmoM2+ Cdk6−/− 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 SmoM2+ Cdk6−/− tumor weight with and without palbociclib, suggesting that CDK4 is not a significant driver of Hh-associated medulloblastoma growth (Figure 3A).

Medulloblastoma acquires resistance to single-agent molecular therapy with vismodegib (19, 20). Further suggesting a possible role for CDK4/6 inhibition in medulloblastoma treat-
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 suggest 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 KO mice. 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 medulloblastoma...
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. DAOY medulloblastoma cells, representative of Hh-associated medulloblastoma, had elevated expression of Gli1, Ptc1, and Cdk6 relative to D283 and D341 medulloblastoma cells, which is representative of group 3 or group 4 medulloblastoma (Figure 3H) (24, 25). Consistently, palbociclib significantly reduced the amount of Ki-67-positive DAOY cells in a dose-dependent manner and only mildly reduced Ki-67 expression in D283 and D341 cells (Figure 3I and Supplemental Figure SD). 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 CDK6, and only mildly reduced Ki-67 expression in D283 and D341 cells (Figure 3I and Supplemental Figure SD). These data suggest 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. PKK 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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11. Lopez-Rios J, et al. GLI3 constrains digit number and only mildly reduced Ki-67 expression in D283 and D341 cells (Figure 3I and Supplemental Figure SD). These data suggest that CDK4/6 inhibition will be an effective therapy for patients with Hh-associated medulloblastoma.