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 GLI1 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.

Introduction

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Hh ligands relieve Patched1 (PTCH1) repression of Smoothened (SMO), allowing SMO to localize to cilia and activate GLI1 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 Glil2-knockin allele that encodes a fusion of GLI2 to EGFP and FLAG tags (Glil2-EGFP) (7). Mice homozygous for the Glil2-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 Glil2-EGFP allele into 2 established mouse models of Hh-associated medulloblastoma. These models make use of Cre recombinase under the control of Math1 regulatory sequences to express a constitutively active, oncogenic point mutant of Smo (SmoM2) or to inactivate Ptch1 (Ptch1) in the EGL (9, 10). In these mouse models, Glil2-EGFP (i) was expressed under endogenous regulatory control, (ii) recapitulated GLI2 activity, interactions, and localization, and (iii) allowed us to immunoprecipitate GLI2 and identify target genes (8).

RNA sequencing of Math1-Cre SmoM2 Glil2-EGFP medulloblastomas demonstrated that, as expected, general Hh target genes, such as Ptchl, Ptcchl, and Glil, and recognized markers of Hh-associated medulloblastoma, including Sfrp1, Pdlim3, and Met, were increased relative to control cerebellums (Figure 1A, Supplemental Figure 1, A and B, and Supplemental Table 1). Other genes not involved in Hh signaling itself were also upregulated, including Cdk6, the expression of which was 167 ± 42-fold higher than that in controls (Figure 1, A and B). Cdk6 encodes cell division kinase 6 (CDK6), which, when bound to cyclin D, phosphorylates retinoblastoma protein (RB) and activates E2F transcription factors to stimulate cell-cycle progression. Like Cdk6 transcript, Cdk6 protein was strongly increased in Hh-associated medulloblastoma (Figure 1C). As (i) Cdk6 is a target of Hh signaling during limb-bud development, (ii) expression of Cdk6 is an independent negative prognostic factor in human medulloblastoma, and (iii) inhibition of Cdk6 in vitro suppresses medulloblastoma cell proliferation, we hypothesized that Cdk6 could be functionally important in Hh pathway-associated medulloblastoma (11-13). Therefore, we compared Cdk6 expression in diverse adult and pediatric human brain tumors and found that Cdk6 was particularly elevated in medulloblastoma (Supplemental Figure 1, C and D). Interestingly, Cdk6 expression was equivalent in all 4 classes of medulloblastoma, suggesting that it may be a common effector
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 SMOOCHED agonist (SAG) (16). Hh pathway activation in this system induced a modest increase in expression of cell cycle effectors, including Ccnd1 and Cdk6 (Figure 1D and Supplemental Figure 2D). Concurrent administration of SAG and an E2F-DNA binding antagonist blocked the Hh pathway–mediated induction of all cell cycle transcripts examined except for 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 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-

Figure 1. Hh signaling induces CDK6 in medulloblastoma. (A) Volcano plot generated by RNA sequencing of the medulloblastomas from 3 P35 Math1-Cre SmoM2c Gli2-EGFP female mice relative to the cerebella of 3 P35 SmoM2c Gli2-EGFP female littermate controls. 2676 genes are differentially expressed with fold-change of 3 or more. P value of less than 0.008, and false discovery rate of 0.05 or less. Hh pathway targets and Cdk6 (red) are enriched in Hh-associated medulloblastoma. (B) Quantitative reverse-transcriptase PCR (qRT-PCR) demonstrates enrichment of Gli1 and enrichment of Cdk6 expression in Hh-associated medulloblastoma (blue) relative to age- and sex-matched littermate cerebella (gray). *P < 0.004, t test. n = 4 mice per genotype, representative of 3 experiments. (C) Immunoblot demonstrates elevated levels of Cdk6, cyclin D1, and phospho-RB proteins in Hh-associated medulloblastoma. n = 2 mice, representative of 3 experiments. (D) qRT-PCR of ciliated NIH/3T3 cells treated either with SAG alone to activate the Hh pathway (green) or in conjunction with HLM006474 to inhibit E2F DNA binding (black). E2F antagonism inhibits induction of all cell cycle effectors in response to Hh stimulation except for Cdk6 and Ccnd1.

*P < 0.05, t test. n = 3, representative of 3 experiments.
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 medulloblastoma model. Homozygous genetic deletion of Cdk6 (Math1-Cre SmoM2 Cdk6KO/KO) reduced the weight (32% ± 2%, Figure 3A) of the tumors (blue) and 3 age- and sex-matched SmoM2 littermate control cerebella (gray). *P < 0.008, t test. Representative of 3 experiments. (F) Activity of the Cdk6 promoter luciferase reporter in NIH/3T3 cells treated with vehicle (gray) or SAG (green). SAG activates the Cdk6 promoter extending from GLI2-binding site 1 to the transcriptional start site (1-Start) or from site 4 to the transcriptional start site (4-Start), but not from site 1 to site 3 (s1–3). *P < 0.01, t test. n = 3, representative of 3 experiments. (G) Activity of the Cdk6 promoter luciferase reporter in NIH/3T3 cells cotransfected with control vector (gray) or a constitutively active GLI2-CLEG plasmid (green). *P < 0.02, t test. n = 3, representative of 2 experiments. (H) Activity of the Cdk6 promoter luciferase reporter in NIH/3T3 cells cotransfected with control vector (gray) or GLI2-CLEG (green) shows GLI2-CLEG activates gene expression from 3 copies of the Cdk6 promoter site 4, but not from 3 tandem mutant copies. *P < 0.001, t test. n = 3, representative of 3 experiments.

Figure 2. GLI2 binds the Cdk6 promoter to activate gene expression. (A) qRT-PCR of NIH/3T3 cells treated vehicle (gray), SAG alone (green) or in combination with the translation inhibitor cycloheximide (black). *P < 0.02, t test. n = 3, representative of 3 experiments. (B) Schematic of Cdk6 promoter showing putative GLI1-binding sites. (C) EGFP ChIP-qPCR from 3 Math1-Cre SmoM2 GlI2-EGFP tumors (blue) and 3 age- and sex-matched SmoM2 GlI2-EGFP littermate cerebella (gray). *P < 0.03, t test. Representative of 3 experiments. (D) Histone ChIP-qPCR from 3 Math1-Cre SmoM2 tumors (aqua) and 3 age- and sex-matched SmoM2 littermate control cerebella (gray). *P < 0.002, t test. Representative of 2 experiments. (E) EGFP ChIP-qPCR from NIH/3T3 cells stably expressing GLI2-EGFP treated with vehicle (gray), SAG alone (green), or SAG in combination with the SMO inhibitor vismodegib (black). *P < 0.008, t test. n = 3, representative of 3 experiments. (F) Activity of the Cdk6 promoter luciferase reporter in NIH/3T3 cells treated with vehicle (gray) or SAG (green). SAG activates the Cdk6 promoter extending from GLI2-binding site 1 to the transcriptional start site (1-Start) or from site 4 to the transcriptional start site (4-Start), but not from site 1 to site 3 (s1–3). *P < 0.01, t test. n = 3, representative of 3 experiments. (G) Activity of the Cdk6 promoter luciferase reporter in NIH/3T3 cells cotransfected with control vector (gray) or a constitutively active GLI2-CLEG plasmid (green). *P < 0.02, t test. n = 3, representative of 2 experiments. (H) Activity of the Cdk6 promoter luciferase reporter in NIH/3T3 cells cotransfected with control vector (gray) or GLI2-CLEG (green) shows GLI2-CLEG activates gene expression from 3 copies of the Cdk6 promoter site 4, but not from 3 tandem mutant copies. *P < 0.001, t test. n = 3, representative of 3 experiments.

To confirm the efficacy of Cdk6 inhibition for medulloblastoma, we treated Math1-Cre PtcH1 mice with a different small molecule CDK4/6 antagonist abemaciclib, which also reduced the size of tumors (14% ± 2%, Figure 3F) mice to the transcriptional start site (4-Start), but not from site 1 to site 3 (s1–3). *P < 0.01, t test. n = 3, representative of 3 experiments. (G) Activity of the Cdk6 promoter luciferase reporter in NIH/3T3 cells cotransfected with control vector (gray) or a constitutively active GLI2-CLEG plasmid (green). *P < 0.02, t test. n = 3, representative of 2 experiments. (H) Activity of the Cdk6 promoter luciferase reporter in NIH/3T3 cells cotransfected with control vector (gray) or GLI2-CLEG (green) shows GLI2-CLEG activates gene expression from 3 copies of the Cdk6 promoter site 4, but not from 3 tandem mutant copies. *P < 0.001, t test. n = 3, representative of 3 experiments.

To confirm the efficacy of Cdk6 inhibition for medulloblastoma, we treated Math1-Cre PtcH1 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 SmoM2 Cdk6KO/KO 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 Cdk6KO/KO 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-
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 c 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 is mediated through a G1 cell cycle arrest and cellular senescence mechanism.
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 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 DAOY 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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