

Cell Reports

Supplemental Information

Casein Kinase 1 δ Is an APC/C^{Cdh1} Substrate that Regulates Cerebellar Granule Cell Neurogenesis

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure S1, Related to Figure 3. Inhibition or Knockdown of CK1 δ Reduces mRNA Levels of SHH-Pathway Components

(A) SR-1277 (100 nM) decreases the expression of *Gli1* and *Gli2* but does not alter that of *Gli3* or *HHip* induced by SHH. GCPs were treated with SHH (75 ng/mL) and/or SR-1277 for 24 or 48 h. The mRNA was then amplified by qRT-PCR, and fold-change in gene expression was determined by normalizing the data to *GAPDH* values relative to control samples. (B) CK1 δ knockdown reduces the expression of *Gli1* and *Gli2* in the presence of SHH. GCPs were electroporated with two different siRNAs against CK1 δ , and mRNA levels were analyzed after 72 h in vitro. Results shown are the averages of three independent experiments and are represented as the mean and standard error of the mean (\pm SEM) (*p <0.05, **p <0.001, ***p <0.001, ****p <0.0001).

Supplemental Figure S2, Related to Figure 4. CK1 δ and Wee1 Levels Are Inversely Related During Mitotic Exit

(A) CK1 δ and Wee1 levels are inversely related. Extracts from asynchronous HeLa cells were transfected with Cdh1, CK1 δ , or GFP siRNAs and analyzed by immunoblotting. The data are representative of three independent experiments. (B) Quantification of protein levels in (A). (C) CK1 δ overexpression reduces Wee1 levels. Asynchronous HeLa cells were transfected with empty vector, CK1 δ -V5, or CK1 δ -V5 Δ DB1 DB2 and then immunoblotted to measure CK1 δ or Wee1 levels. (D) Quantification of protein levels in (C). (E) CK1 δ levels decrease upon the cells' release from mitosis. HeLa cells were synchronized in

mitosis by sequential treatment with thymidine and nocodazole. The cells were then released from arrest with nocodazole and collected at the indicated time points. Cell extracts were prepared and either immunoblotted for the indicated proteins (E) or analyzed by flow cytometry (G). (F) Quantification of protein levels in (E).

Supplemental Figure S3, Related to Figure 9. CK1 δ Is a Potential Therapeutic Target in Murine Medulloblastoma

(A) *Csnk1d* (CK1 δ) and *Wee1* mRNA levels do not vary in *Ptch1*^{+/-}, *Cdkn2*^{-/-}, *Trp53*^{-/-}, or *Myc*-driven tumors compared to those expressed in normal GCPs. Results shown are the averages of three independent experiments and are represented as the mean \pm SEM. (B) Microarray analyses of *Csnk1d* (CK1 δ) mRNA expression in human subgroups and mouse models of medulloblastoma show no differences between the disease subgroups. Data were analyzed by ANOVA using Partek[®] Genomics Suite[™] 6.6. The highest-expressing probe set under the *Csnk1d* gene was tested in each data set. (C) *Emi1* levels are higher in *Myc* models of medulloblastoma than in normal GCPs. (D) Quantification of (C). (E) CellTiter-Glo assays were performed on DAOY cells treated with increasing concentrations of the indicated compounds for 24 h to measure their ATP content. (F) Comparison of the effects of SR-1277 and various compounds on the ATP content of D283 cells.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animal Husbandry

Csnk1d^{fl/fl} mice were purchased (Jackson Laboratory). *Tg(Atoh1-Cre)* mice were generously provided by Dr. David Rowitch. *Fzr1^{fl/fl}* mice were bred with *Tg(Atoh1-Cre)⁺* mice to obtain *Tg(Atoh1-Cre)⁺;Fzr1^{fl/fl}* and *Tg(Atoh1-Cre)⁻;Fzr1^{fl/fl}* animals. Similarly, *Csnk1d^{fl/fl}* (Jackson laboratory, strain 010487) were crossed with *Tg(Atoh1-Cre)* mice to obtain *Tg(Atoh1-Cre)⁺;Csnk1d^{fl/fl}* and *Tg(Atoh1-Cre)⁻;Csnk1d^{fl/fl}* animals. These mice were then used to obtain GCP cells and organotypic cultures. All mice were housed in an American Association of Laboratory Animal Care–accredited facility and were maintained in accordance with NIH guidelines. This study was approved by the Institutional Animal Care and Use Committees of the University of Miami, The Rockefeller University, Scripps Florida, and St. Jude Children’s Research Hospital.

GCP Isolation and Compound Treatment

GCPs were purified from cerebellar cortex of postnatal day (P) 6 CD-1 mice (Jackson Laboratory), *Tg(Atoh1-Cre)⁺;Csnk1d^{fl/fl}* mice, and *Tg(Atoh1-Cre)⁻;Fzr1^{fl/fl}* mice by using Percoll gradient sedimentation to produce an enriched GCP fraction. The cells were then preplated on a Petri dish to remove contaminating glia. Purified GCPs were resuspended in medium [DMEM/F12, 1.5% glucose, 20 mM glutamine, 10% horse serum, 5% fetal bovine serum (FBS)] and then plated at a density of 3×10^6 cells/well in 6-well dishes or 3×10^5 cells/well in 96-well plates. For proliferation assays, GCPs were maintained in culture; for cell cycle exit and differentiation assay, GCPs were plated in poly-D-lysine/laminin–coated plates. For drug treatment, 100 nM of SR-653234 or SR-1277 or 20 μ M of D4476 or DMSO was added to the culture medium for 24 or

48 h. Human recombinant SHH (75 ng/mL; Applied Stem Cell) was added. Subsequently, GCPs were either used for the ³H-thymidine–incorporation assay or fixed for FACS analysis, or they were lysed to obtain protein for Western blot analysis or RNA for qRT-PCR.

Apoptosis Assays

Purified GCPs were plated at 3×10^5 cells/well in 96-well plates on poly-D-lysine/laminin–coated plates for 24 h. SR-653234 (100 nM), SR-1277 (100 nM), or DMSO as a control was then added for 24 h. SYTOX (5 nM; Invitrogen) was used to stain apoptotic cells, and Hoechst stain (Invitrogen) was used to label nuclei. Both were added to the culture medium for 10 min at room temperature. Images and processing to obtain the percentage of dead cells was performed with the Cellomics apparatus.

Electroporation of GCPs

Isolated GCPs were electroporated with the Amaxa Mouse Neuron Nucleofector[®] Kit (Lonza) and Amaxa Nucleofector Machine per the manufacturer's instructions. A total amount of 0.75 nmol of siRNA or 30 µg of vector were used to electroporate 3 to 5×10^6 purified GCPs. The following siRNAs were used: negative siRNA (Neg. siRNA; Thermo Fisher Scientific, Cat # D-001810-01-05), CK1δ (CK1δ #1; Ambion, Cat # 4390815, ID s98267), CK1δ (CK1δ #2; Ambion Cat 4390815, ID s98268), and CK1ε (Ambion, Cat # 4390815, ID s77692). The following vectors were used: V5 empty vector and CK1δ-V5.

EdU-Proliferation Assay

After 72 h in culture, GCPs were treated with EdU (20 μ M) for 2 h. The cells were then washed with PBS, plated in poly-D-lysine (100 μ g/mL)-laminin (20 μ g/mL)-coated dishes for 2 to 3 h, and further fixed with 4% paraformaldehyde/30% sucrose. Cells were permeabilized and stained using the Click-iT[®] EdU Alexa Fluor[®] 594 Imaging Kit (Invitrogen). GCP aggregates were imaged using the z-stack of a confocal laser-scanning microscope (Olympus, FV1000), and the images were analyzed using Fiji software (ImageJ).

³H-Thymidine Assay

GCPs were plated (3×10^5 cells/well) in 96-well dishes. For GCPs obtained from CD1 mice, DMSO, SR-653234 (100 nM), or SR-1277 (100 nM) was added to the medium, and cells were maintained in culture for 24 h. GCPs from *Tg(Atoh1-Cre);Csnk1d^{fl/fl}* mice were maintained in the presence of SHH (75 ng/mL) for 24 h. Then, 1 μ Ci [methyl-³H]-thymidine (Amersham) was added to each well; the cells were harvested 22 h later and analyzed using TopCount (Perkin Elmer).

Plasmids, siRNAs, and Site-directed Mutagenesis

The CK1 δ -V5 construct was generated by cloning the full-length *Csnk1d* (*CK1 δ*) gene from the Gateway donor vector pDONR223-CSNK1D (Addgene, plasmid 23796) into the Gateway destination vector pcDNA-DEST40 (Invitrogen, Cat #

12274-015). This added a C-terminal V5-epitope tag to CK1 δ . Flag-Cdh1 and Flag-K328M-Wee1 constructs were derived from pCS2+-based plasmids. The following siRNAs were used in this study: GFP (GFP-22 siRNA, 5 nmol, Qiagen, Cat # 1022064), CK1 δ (Hs_CSNK1D_5 FlexiTube siRNA, 5 nmol, Qiagen, Cat # S100287406), CK1 δ (ONTARGETplus Human CSNK1D siRNA, 5 nmol, Thermo Scientific, Cat # L-003478-01-0005), Cdh1 (Hs_FZR1_1 FlexiTube siRNA, 5 nmol, Qiagen, Cat # S100114905), Cdh1 (ON-TARGETplus Human FZR1 siRNA, 5 nmol, Thermo Scientific, Cat # L-015377-00-0005). Site-directed mutagenesis was performed using the Quick Change II Site-Directed Mutagenesis Kit (Agilent Technologies, Cat # 200523) per the manufacturer's instructions. The following primers were used for the mutation of CK1 δ D-boxes:

DB1

Forward primer: 5'-gagagtcggaacgcgtaccgggcccggccggaagatc-3'

Reverse primer: 5'-gatcttccgcccggcgtacgcgttcccgactctc-3'

DB2

Forward primer: 5'-cttgaattgaacaatcccagcagatgacgcggagtctctgggctacg-3'

Reverse primer: 5'-cgtagcccagagactccgcgtcatctgctcgggattgttcaattccaag-3'

HeLa Cell Culture System, Transfection, and Cell Synchronization

HeLa cells were maintained in culture at 37 °C and 5% CO₂ in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Plasmids were transfected using *TransIT*[®]-LT1 Transfection Reagent (Mirus Bio, Cat # MIR 2304) per the manufacturer's instructions. The siRNAs were transfected with DharmaFECT 1 Transfection Reagent (Thermo Scientific, Cat # T-2001-02) per

the manufacturer's instructions.

For the mitotic-exit measurements, we treated adherent HeLa cells with thymidine (2 mM) for 16 h, washed the cells with PBS, and released them into thymidine-free medium for 3 h. The cells were then treated with nocodazole (330 nM) for 12 h. Cells arrested in mitosis were collected by mitotic shake-off, washed with PBS, and released into nocodazole-free medium.

Flow Cytometry

For flow cytometric analysis, we isolated cells from dishes, washed them with PBS and 1% BSA, and fixed them in a solution of PBS (1%), BSA (10%), and ethanol overnight at 4 °C. Cells were then stained with 69 µM propidium iodide in 38 µM sodium citrate buffer and 1 µM RNase A at 37 °C for 30 min. The number of cells in G1, S, or G2 phases was determined using a fluorescence-activated cell-sorting device (LSRII, Becton Dickinson) and analyzed by FlowJo software.

Cycloheximide Degradation Assay

Cycloheximide (100 µg/mL) or DMSO was added to HeLa cells 2 days after they were either transfected with plasmids or siRNAs or treated with compounds. Cells were then harvested at specific time points, and extracts were prepared (as described above) for SDS-PAGE and Western blot analyses.

In vitro Degradation and Ubiquitination Assays

To prepare G1 HeLa cell extracts for in vitro degradation and ubiquitination assays, we synchronized cells with a thymidine/nocodazole block (as described above) and released them into nocodazole-free medium for 4 h; cells synchronized at G1. For the degradation assay, we washed the cells with PBS and resuspended them in swelling buffer (20 mM HEPES at pH 7.7, 5 mM MgCl₂, 5 mM KCl, 1 mM DTT, 1X protease inhibitor cocktail, 1 μM microcystin-LR) at a ratio of 1 mL packed cells to 0.75 mL buffer.

Cells were lysed by the freeze-thaw method (liquid nitrogen/37 °C water bath) and then passed through a 20.5-gauge needle twice. The resulting lysate was cleared by centrifugation at 20,000 x g for 20 min at 4 °C. Immediately before use, the lysate was mixed with a 1:1:1 ratio cocktail of energy mix (7.5 mM creatine phosphate, 2 mM ATP, 1 U rabbit muscle creatine phosphokinase, 0.1 mM EGTA at pH 7.7, 1 mM MgCl₂), cycloheximide (100 μg/mL), and ubiquitin (500 μM) on ice. In vitro translated, ³⁵S-labeled substrate (1 μL) was added to the mix. In vitro translation was performed using the TNT Quick Coupled Transcription/Translation System (Promega, Cat # L1170) in the presence of 20 μCi [³⁵S] methionine (EXPRESS Protein Labeling Mix, Perkin Elmer, Cat # NEG072014MC). Reactions were initiated by shifting the tubes to 37 °C. Samples were collected at various time points and mixed with Laemmli sample buffer to stop the reaction. They were boiled at 92 °C for 5 min, resolved by SDS-PAGE, and analyzed by autoradiography.

For the ubiquitination assays, cells were washed with PBS and resuspended in lysis buffer (Tris-HCl at pH 7.7, 150 mM NaCl, 20 mM β-glycerophosphate, 5 mM MgCl₂, 1 mM NaF, 1 mM DTT, 0.2% IGEPAL CA-630, 10% glycerol, 1X protease inhibitor cocktail, and 1 μM microcystine-LR). Cells

were lysed, and the resulting lysate was cleared as described above. Anti-Cdc27 antibodies were coupled to rProtein A Sepharose beads (GE Healthcare, Cat # 17-1279-01), and the beads were mixed with cell lysates at a ratio of 1:10 for 2 h at 4 °C.

After incubation, the beads were washed with TBS plus 0.01% Tween-20 and resuspended in 1X TBS. The beads were mixed on ice with 200 nM His6-UBE1, 200 nM His6-UbcH10, 200 nM UbcH5a, 500 µM ubiquitin, 5 µM ubiquitin aldehyde, 50 µM MG-132, 7.5 mM creatine phosphate, 2 mM ATP, 1 U rabbit muscle creatine phosphokinase, 0.1 mM EGTA at pH7.7, and 1 mM MgCl₂. In vitro translated, ³⁵S-labeled substrate (1 µL) was added to the mix, and ubiquitination was initiated by shifting the tubes to 37 °C. Samples collected at various time points were mixed with Laemmli sample buffer, boiled at 92 °C for 5 min, resolved by SDS-PAGE, and analyzed by autoradiography. Recombinant CK1δ (instead of ³⁵S-labeled CK1δ) was used in an identical reaction in Figure 4F.

Protein Extract Preparation, Antibodies, and Western Blot Analysis

Cells were homogenized, and extracts were prepared using lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1X protease inhibitor cocktail, 1 µM microcystin-LR). Cells were lysed by the freeze-thaw method (liquid nitrogen/37 °C water bath) and further sonicated. The soluble fraction was recovered by centrifugation at 20,000 x g for 20 min at 4 °C. Protein concentration was measured with the BCA Protein Assay Kit (Pierce Biotechnology), and 30 µg of protein from each sample was resolved by SDS-PAGE. The resolved bands

were transferred onto a nitrocellulose membrane and subjected to Western blotting with the appropriate antibodies.

The following primary antibodies were used: anti-CK1 δ antibody (C-8) from Santa Cruz Biotechnology (Cat # sc-55553), anti-CK1 γ 2 antibody (26-P) from Santa Cruz Biotechnology (Cat # sc-130365), anti-CK1 α antibody (C-19) from Santa Cruz Biotechnology (Cat# sc-6477), anti-Skp1 p19 antibody (H-163) from Santa Cruz Biotechnology (Cat # sc-7163), anti-phospho-Histone H3 (Ser 10)-R antibody from Santa Cruz Biotechnology (Cat # sc-8656-R), anti-phospho-Cdc2 (Tyr15) antibody from Cell Signaling (Cat # 9111S), anti-Cyclin B1 [V152] antibody from Abcam (Cat # ab72), and anti-CK1 ϵ antibody from BD Transduction Laboratories (Cat # 610445).

The following secondary antibodies were used: anti-mouse IgG-HRP antibody from GE Healthcare (Cat # NXA931) and anti-rabbit IgG-HRP antibody from GE Healthcare (Cat # NA9340V).

RNA Isolation and qRT-PCR

Cells samples were lysed in 1 mL TriZol Reagent (Invitrogen), and the RNA was purified with the RNeasy Mini Kit (Qiagen). RNA was then reverse-transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan probes were designed with the TaqMan Gene Expression Assay tool (Applied Biosystems). The qRT-PCR was performed using a TaqMan[®] Gene Expression Master Mix (Applied Biosystems) in a CFX384 Touch[™] Real-Time PCR Detection System (Bio-Rad). Fold-change in gene expression was estimated using the computed tomography comparative method and

normalizing to the GAPDH computed tomography values and relative to control samples.

Organotypic Slice Cultures and Proliferation Assays

Cerebella were isolated from P8 mice. Sagittal slices (250- μ m) of cerebellar cortex were generated using a Leica VT1000S vibratome, and slices were plated on Millipore culture inserts in 6-well culture dishes (Falcon) containing 1.5 mL serum-free medium (Basal Medium Eagle (Gibco), 0.45% D-(+)-glucose solution (Sigma), 1X ITS supplement (Sigma), 2 mM L-glutamine (Gibco), 100 U/mL penicillin/streptomycin (Gibco). The slices were then submerged in 2.5 mL medium containing DMSO and SR-653234, SR-1277, or D4476 for 1 h, after which 1 mL was removed so that the slices were no longer submerged, and the medium was below the insert.

For the proliferation assays, we added 25 μ M EdU (1 mL; Invitrogen) on top of the slices after they were in culture for 22 h. Thus, the final concentration was 10 μ M EdU per 2.5 mL medium. After 1 h, we removed 1 mL of medium so that the cells were no longer submerged. Twenty hours later, the slices were fixed for 2 h with 4% paraformaldehyde/PBS and then incubated in 30% sucrose/PBS overnight at 4 °C. The slices were freeze-thawed on dry ice, washed with PBS, permeabilized, and blocked in a solution of 0.5% Triton X-100, 10% normal goat serum, and PBS for 3 h at room temperature. The slices were then washed with PBS and 3% BSA/PBS and processed with the Click-iT kit (Invitrogen) for EdU detection. The slices were finally washed with 3% BSA/PBS, incubated with 1/1000 DRAQ5 in PBS (Biostatus Limited) to stain the nuclei, and mounted with ProLong[®] Gold Antifade mounting medium

(Invitrogen).

Immunohistochemistry

Cerebella were fixed with 4% paraformaldehyde/PBS for 48 h, embedded in 30% sucrose in PBS, and cut into sections (20- μ m thick) with a cryostat (Leica). Sections were then permeabilized and blocked in a solution of 5% Triton X-100, 5% normal goat serum, and PBS for 1 h at room temperature and incubated with primary antibodies (goat anti-CK1 δ (1/200; Abcam) and anti-calbindin (1/1000; Swant) overnight at 4 °C. The slices were then washed with PBS, and secondary antibodies (Alexa Fluor 555 donkey anti-goat IgG; Alexa Fluor 488 goat anti-rabbit IgG) were added consecutively for 2 h at room temperature. Sections were then washed with PBS and mounted using ProLong Gold Antifade mounting medium (Invitrogen). Confocal images were acquired with a confocal laser-scanning microscope and were further analyzed with Fiji software (ImageJ).

In vivo Allografts

Frozen *Ptch1*^{+/-} tumor cells were thawed, washed with PBS, and injected subcutaneously into the right flank of a NU-Foxn1nu mouse (Charles River Laboratories). When the *Ptch1*^{+/-} tumor grew to 1 \times 10³ mm³, the tumor was removed and placed in PBS. It was then passed through a metal strainer, washed twice with cold PBS, washed twice with cold DMEM (Invitrogen, Cat # 11965), and resuspended in DMEM. Matrigel (1 mg/mL; BD Biosciences, Cat #

354248) was added to the cell solution. Mice (n=8) were injected subcutaneously in the right flank with 200 μ L of the cell solution (5×10^6 cells per mouse). Tumor sizes were measured with digital calipers daily, recorded, and used for tumor volume calculations. Treatment with SR-1277 began when tumors reached 50 to 90 mm^3 . Intraperitoneal injections were administered twice daily at a concentration of 10 mg/kg for 8 days.

Mouse G3 Medulloblastoma Neurospheres in Culture

Tumor cells were maintained in culture as previously described (Kawauchi et al., 2012). Briefly, tumor cells were grown in low-attachment flasks in Neurobasal medium (Invitrogen, Cat # 21103-049), B27 (Invitrogen, Cat # 175-04-044), N2 (Invitrogen, Cat # 175-02-048), 30% BSA solution (Sigma, Cat # A9576-50ml) and 100 μ g/mL of the factors Human bFGF (Peprotech, Cat # AF-100-18B) and Human EGF (Peprotech, Cat # AF-100-15). Growth factors were added every 2 to 3 days.

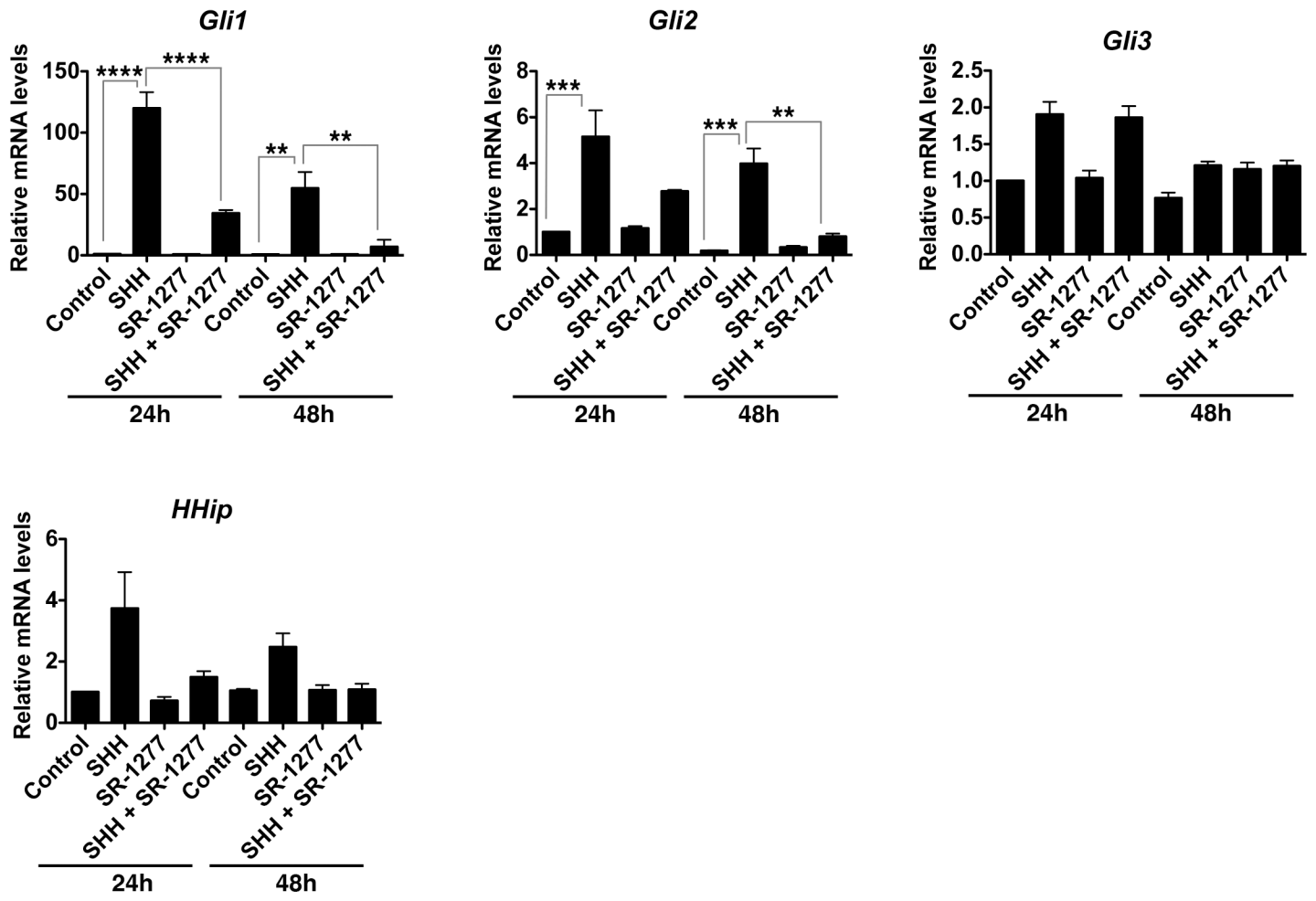
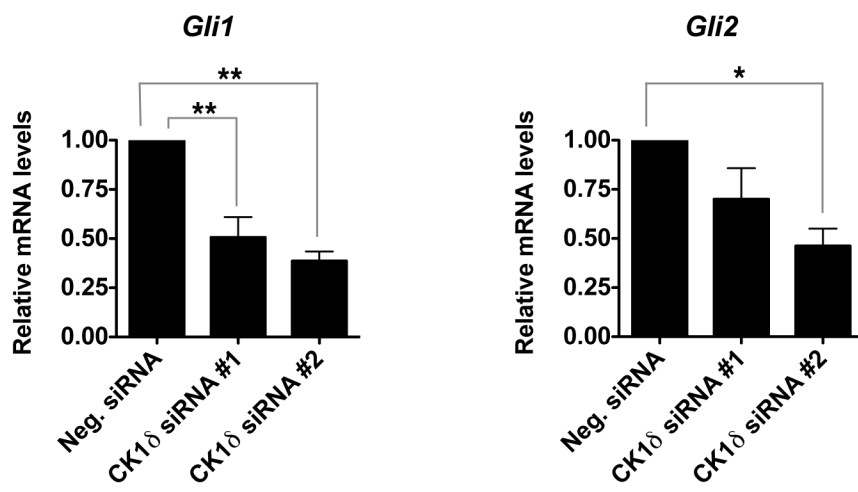
Transduction of DAOY Cells with Luciferase-Expressing Lentivirus

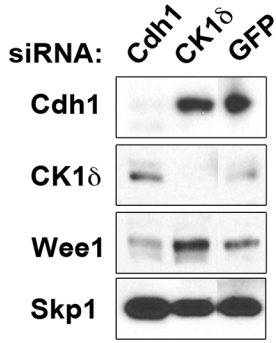
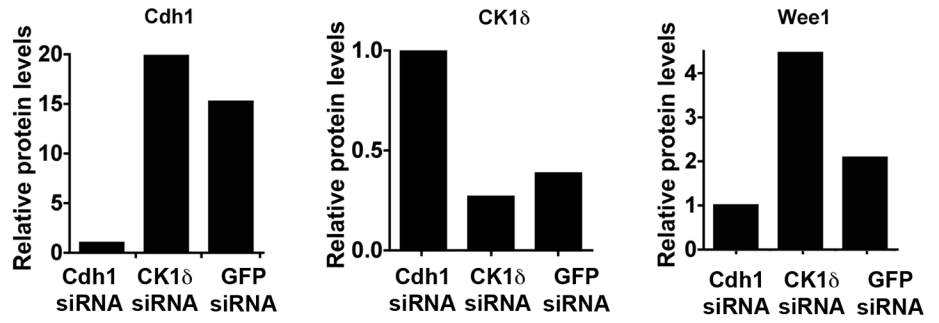
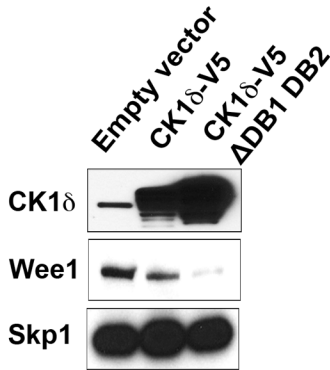
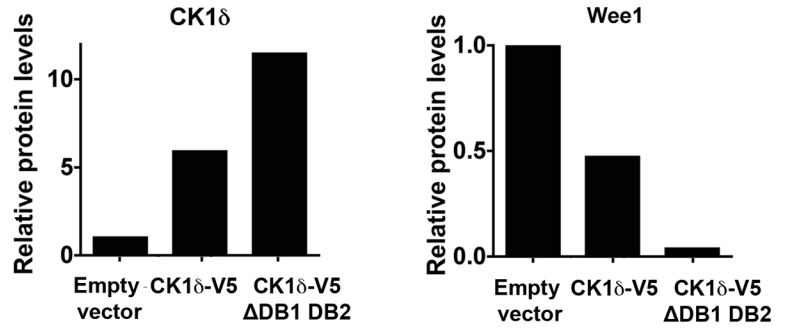
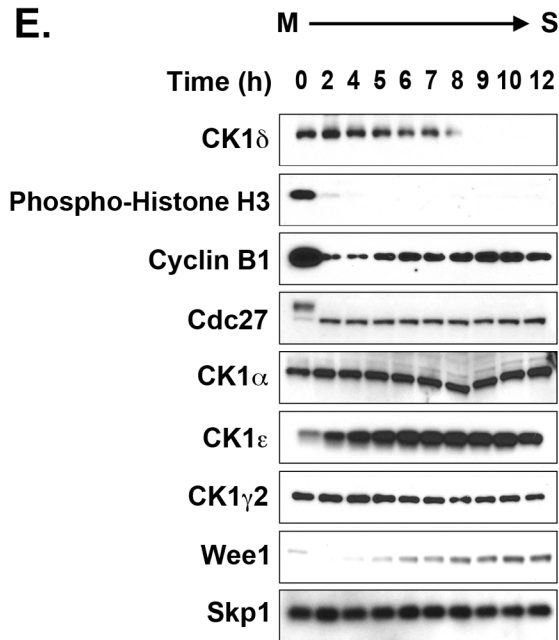
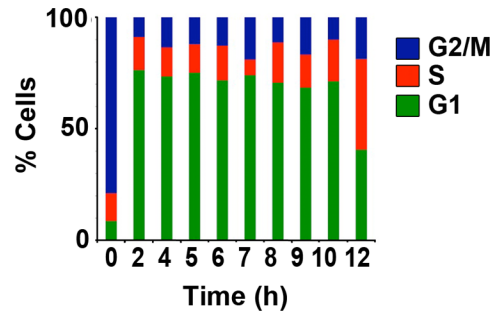
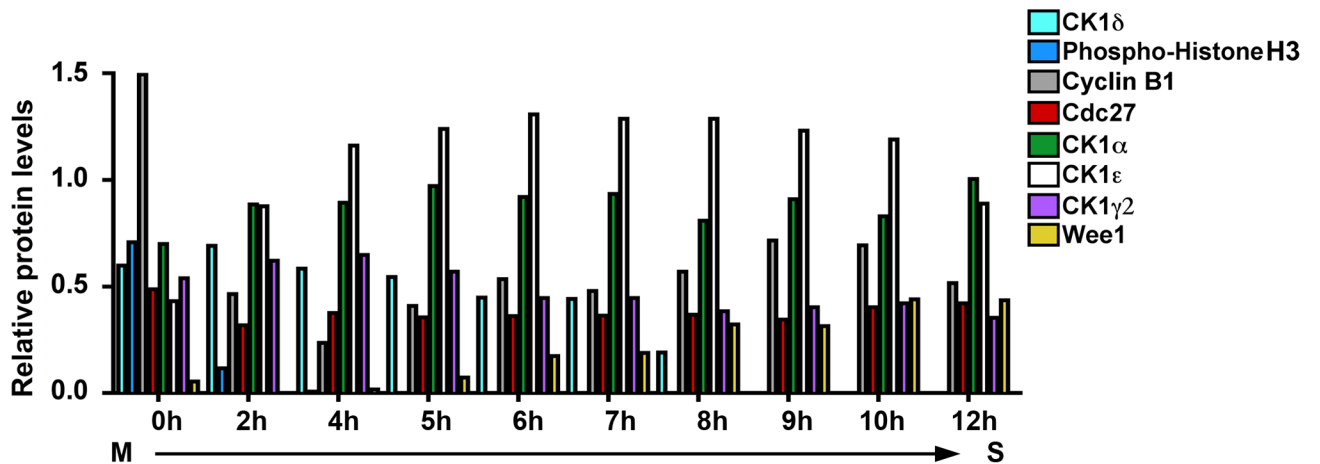
DAOY cells (ATCC) were transduced with Firefly luciferase lentivirus (Capital Biosciences). Cells were plated at a density of 5×10^4 cells/well in a 24-well plate. The medium was replaced with 0.5 mL virus suspension (10^8 U/mL) in the presence of 8 μ g/mL polybrene (Life Technologies). After a few passages, the transduced cells were selected with 1 μ g/mL puromycin to obtain a stable transduction. After 2 weeks, cells were analyzed for luciferase expression with the luciferase reporter gene assay system Britelite (Perkin Elmer). The EnVision[®] Multilabel Reader (Perkin Elmer) was used to measure the

luminescence signal produced by the cells.

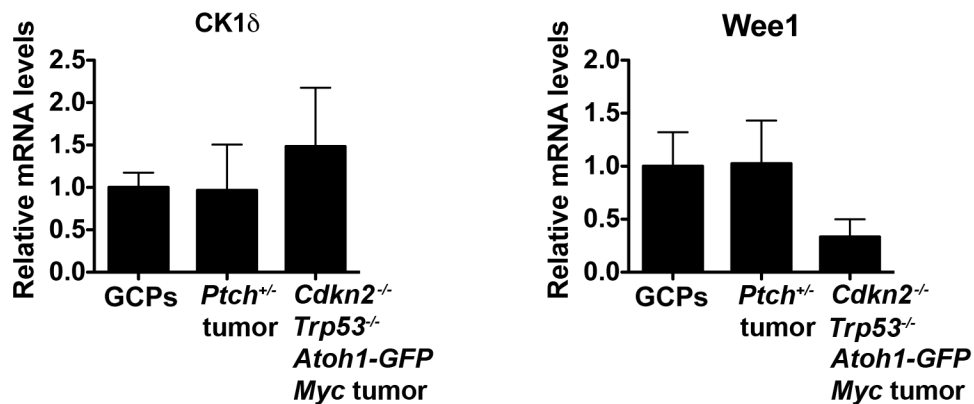
Imaging of Luciferase-expressing DAOY Cells Transplanted Intracranially

NCr nude mice (Taconic Biosciences) were anesthetized, and a sagittal incision over the parieto-occipital bone was performed. A 25-gauge needle was used to puncture the skull at 2 mm to the right of the bregma and 1 mm anterior to the coronal suture. Using a 26-gauge Hamilton syringe, we injected 10^5 cells at 3-mm depth into the brain. Ten days after the transplantation procedure, mice received an intraperitoneal injection of D-luciferin (150 mg/kg; Caliper Life Sciences), were anesthetized with isoflurane, and imaged with the IVIS Spectrum Imaging System (Xenogen) for 10 to 120 s. Tumor growth was monitored weekly. To quantify the bioluminescence, we encircled the regions of interest on every image to include the entire tumor in each animal. The integrated flux of photons (photons per second) within each region of interest was determined using Living Image[®] software (Xenogen).

A.**B.****Figure S1**

A.**B.****C.****D.****E.****G.****F.****Figure S2**

A.



B.

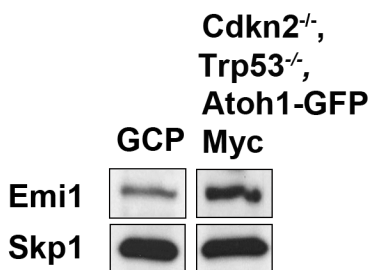
Human medulloblastoma

Gene	pValue_subgroup_ANOVA	Mean_G3	Mean_G4	Mean_shh	Mean_Wnt
<i>Csnk1d</i>	0.00032402	9.42393	9.52895	8.9672	9.23074

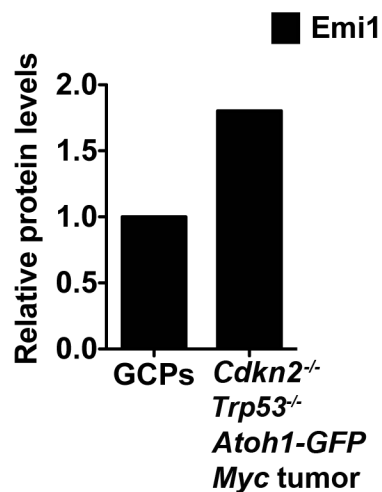
Mouse medulloblastoma

Gene	pValue_model_ANOVA	Mean_Myc	Mean_Ptc_ShH	Mean_trp53_shh	Mean_Wnt
<i>Csnk1d</i>	7.86E-06	10.7413	11.1746	11.4815	11.9132

C.



D.



E.

DAOY

Compounds	IC50 (μM)
SR-1277	1.124
MK-2206	1.742
BKM-120	1.58
GDC0941	4.481
MK-1775	0.6724

F.

D283

Compounds	IC50 (μM)
SR-1277	0.6518
MK-1775 + SR-1277	0.5364
MK-1775	0.6178
GDC-0449	1.697
GDC-0449 + SR-1277	0.6582

Figure S3