HDM2 promotes WIP1-mediated medulloblastoma growth

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Medulloblastoma is the most common malignant childhood brain tumor. The protein phosphatase and oncogene WIP1 is over-expressed or amplified in a significant number of primary human medulloblastomas and cell lines. In the present study, we examine an important mechanism by which WIP1 promotes medulloblastoma growth using in vitro and in vivo models. Human cell lines and intracerebellar xenografted animal models were used to study the role of WIP1 and the major TP53 regulator, HDM2, in medulloblastoma growth. Stable expression of WIP1 enhances growth of TP53 wild-type medulloblastoma cells, compared with cells with stable expression of an empty-vector or mutant WIP1. In an animal model, WIP1 enhances proliferation and reduces the survival of immunodeficient mice bearing intracerebellar xenografted human medulloblastoma cells. Cells with increased WIP1 expression also exhibit increased expression of HDM2. HDM2 knockdown or treatment with the HDM2 inhibitor Nutlin-3a, the active enantomer of Nutlin-3, specifically inhibits the growth of medulloblastoma cells with increased WIP1 expression. Nutlin-3a does not affect growth of medulloblastoma cells with stable expression of an empty vector or of mutant WIP1. Knockdown of WIP1 or treatment with the WIP1 inhibitor CCT007093 results in increased phosphorylation of known WIP1 targets, reduced HDM2 expression, and reduced growth specifically in WIP1 wild-type and high-expressing medulloblastoma cells. Combined WIP1 and HDM2 inhibition is more effective than WIP1 inhibition alone in blocking growth of WIP1 high-expressing medulloblastoma cells. Our preclinical study supports a role for therapies that target WIP1 and HDM2 in the treatment of medulloblastoma.

Keywords: HDM2, MDM2, medulloblastoma, PPM1D, WIP1.

Patients who receive a diagnosis of medulloblastoma, the most common malignant brain tumor of childhood, are currently treated on the basis of disease stage, age at diagnosis, and extent of resection with use of a combination of surgery, chemotherapy, and ionizing radiation (IR).1 Advances in neurosurgical and medical treatments for medulloblastoma have dramatically improved the cure rate. However, disease progression or recurrence is still fatal for up to one-third of patients. Furthermore, patients who are cured often still experience chronic toxicities of treatment that can permanently inhibit appropriate growth, cognition, and motor functions, significantly detracting from their quality of life.

Initial efforts aimed at improving understanding of medulloblastoma tumor biology focused on disease classification with use of cytogenetic aberrations identified by comparative genomic hybridization (CGH).2,3 Gain of the long arm of chromosome 17 (17q) and isochromosome 17q (i17q), consisting of 17p deletion with duplication of 17q, has repeatedly been identified as the most common cytogenetic lesion affecting medulloblastoma in 30%–50% of cases.4–6 More recent attempts to categorize medulloblastoma have focused on the gene expression profile. Two recent publications have segregated medulloblastoma into 4 or 5 subtypes, based on differential expression of genes involved in Wingless (WNT) signaling (group A), Sonic-Hedgehog (SHH) signaling (group B), neuronal differentiation (groups C–D), or expression of photoreceptor genes (groups D–E). Alterations of chromosome 17 and upregulation of genes associated with tumor metastasis were strongly associated with medulloblastomas in subtypes C and D.7,8 Our analysis of this published data suggests
increased expression of the TP53-induced proto-oncogene WIP1 (wild-type TP53-induced phosphatase 1 or protein phosphatase, magnesium-dependent 1, delta, PPM1D), located at chromosomal locus 17q22-23, in group C and D medulloblastomas.

TP53, on chromosome 17p13, is one of the most studied and best-characterized tumor suppressor genes. WIP1 has been implicated as an important regulator of the activity of TP53. In normal cells exposed to environmental stressors, such as ionizing radiation, WIP1 functions in a negative feedback loop with TP53. TP53 induces expression of WIP1, which in turn inactivates and limits the activity of TP53 directly by dephosphorylating serines 15 and 20 and indirectly through dephosphorylation of p38 MAPK and HDM2. Additionally, WIP1 has been shown to cooperate with oncogenes, including c-myc, Ras, and E1A, to transform rodent embryonic fibroblasts. Amplification of 17q22-23 has been described in malignancies, including breast and ovarian carcinomas and in neuroblastoma, most of which are wild-type for TP53. We previously reported that 51% of human medulloblastomas have amplification or gain of WIP1. All primary medulloblastomas that were identified as WIP1-amplified by fluorescence in situ hybridization (FISH) were also amplified by comparative genomic hybridization and exhibited elevated WIP1 mRNA and protein expression, compared with fetal brain controls. Other investigators have demonstrated nuclear staining for WIP1 in 88% of human medulloblastomas. This suggests that WIP1 plays an important role in medulloblastoma tumorigenesis.

In the current study, we showed that stable expression of WIP1 enhances growth of TP53 wild-type medulloblastoma cells, compared with cells with stable expression of an empty vector or mutant WIP1. WIP1 enhances proliferation and reduces the survival of immunodeficient mice bearing intracerebellar xenografts of WIP1 high-expressing human medulloblastoma cells. Medulloblastoma cells with increased WIP1 expression also exhibit increased expression of HDM2. Knockdown or treatment with the HDM2 inhibitor Nutlin-3a, the active enantiomer of Nutlin-3, specifically inhibits the growth of medulloblastoma cells with increased WIP1 expression. Knockdown of WIP1 or treatment with the WIP1 inhibitor CCT007093 results in increased phosphorylation of known WIP1 targets, reduced HDM2 expression, and reduced growth specifically in WIP1 wild-type, high-expressing medulloblastoma cells. Combined WIP1 and HDM2 inhibition is more effective than WIP1 inhibition alone in blocking the growth of WIP1 high-expressing medulloblastoma cells. This suggests that WIP1 and HDM2 are important targets for the treatment of medulloblastoma.

Materials and Methods

Gene Expression Analysis

WIP1 (PPM1D) expression in human medulloblastomas was determined by downloading .cel files from datasets GSE10327 and GSE21140 from the National Institutes of Health Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/). The GSE10327 dataset was generated using an Affymetrix Human Genome U33 Plus 2.0 Array. Prior to analysis, raw data were normalized using a Robust MultiChip Average algorithm. Classification of the 62 medulloblasto samples was maintained as previously reported. Partek Genomics Suite software (Partek) was used to confirm differential WIP1 expression among medulloblastoma groups A–E. Because data in the GSE21140 dataset were generated using an Affymetrix Human Exon 1.0 ST Array, raw .cel files were imported into Partek Genomics Suite and normalized using the Robust MultiChip Average algorithm. Classification of the 103 medulloblastoma samples was maintained according to the original published report.

Gene expression was estimated by averaging the signals for all exons corresponding to the WIP1 (PPM1D) gene. WIP1 expression was compared among all groups (WNT, SHH, group C, and group D).

Materials

Nutlin-3a (Cayman Chemicals) was prepared at a 3.2 mM stock concentration in ethanol and diluted in culture media to 4–8 μM for use in experiments. CCT007093 (Sigma-Aldrich) was prepared at a stock concentration of 5 mM in DMSO and diluted in culture media to 0.05–5 μM for use in experiments. The maximum concentration of ethanol and of DMSO in both drug-treated and control experiments was 0.5% and 0.1%, respectively.

Cell Culture

We routinely maintained human medulloblastoma cell lines, including D556, Med8A, D425, and Daoy in DMEM or modified Eagle’s medium (Invitrogen) with high glucose, 2 mM L-glutamine, and 10% (vol/vol) heat-inactivated FBS at 37°C in 5% CO2. D556 and D425 cell lines were gifts from Tobey MacDonald (Emory University); Med8A cells were obtained from John Kim (Kaiser Permanente), and Daoy parental cells were purchased from ATCC. We generated D556 and Daoy stable-expressing clones by transfecting these cells with an empty vector (pcDNA3/pcDNA), WIP1, or phosphatase-deficient, nonfunctional WIP1-D314A cDNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations and selecting with 200 μg/mL G418 (Sigma-Aldrich). After colonies were identified and growing in selection media, each colony was trypsinized in a sterile cloning cylinder (Bellco Glass) and transferred into a 24-well plate containing 200 μg/mL G418. Cell clones were allowed to grow until almost confluent and were gradually moved into larger cell culture dishes. After clones were split into 10 cm dishes, cell pellets were collected from each clone. Total RNA was extracted, and real-time reverse-transcription polymerase chain reaction (RT-PCR),
described below, was conducted on each clone to determine relative WIP1 expression.

All cell lines were split twice weekly and were screened for mycoplasma contamination every 6 months (Mycoplasma Detection Kit; Lonza Group). WIP1- and phosphatase-deficient WIP1-D314A plasmids were gifts from Lawrence Donehower (Baylor College of Medicine). All cells were passaged for less than 6 months after receipt or resuscitation.

Effects on growth of adherent cells were assessed by plating 1 × 10^5 D556 or Daoy stable clones on 6-well plates in triplicate and harvesting cells with 0.25% trypsin EDTA (Invitrogen) at 24–96 h after initial plating. Cells were counted by trypan blue exclusion using standard methods.

Western Blotting

Cells were extracted from culture plates by scraping or with 0.25% trypsin EDTA (Invitrogen). Cell pellets were washed in phosphate-buffered saline (PBS), centrifuged at 200 × g, and stored at −80°C. Proteins were extracted from cell pellets using RIPA buffer (Cell Signaling), quantified using a standard Bradford assay, and electrophoretically separated on polyacrylamide denaturing gels, as previously described. Protein was transferred onto nitrocellulose membranes and immunoblotted with the designated antibodies as previously described. Antibodies used included WIP1 (Bethyl), phospho-p53 (Ser15; Cell Signaling), p53 (DO-1; Santa Cruz), HDM2 (2A10; Millipore), Phospho-p38 MAPK (Thr180/Tyr182; Cell Signaling), p38 MAPK (EMD4Biosciences), β-actin (AC-15; Sigma-Aldrich), and Vinculin (Sigma-Aldrich). Secondary antibodies Alexa Fluor 680 goat anti-mouse IgG (Invitrogen) or IRDye 800 goat anti-rabbit IgG (Rockland) were used at a dilution of 1:5000 and applied according to the manufacturer’s recommendations. Immunooblots were imaged and the intensity of bands on Western blots was quantified using an Odyssey infrared imaging system (Li-COR Biosciences).

Anchorage-Independent Growth Assays

A total of 8 × 10^4 cells per well were plated in triplicate in 6-well dishes in 1 mL of matrigel (BD Biosciences) diluted 3:1 (media to matrigel). The matrigel cell suspension was allowed to solidify for at least 2 h at 37°C. For Nutlin experiments, after the matrigel solidified, 1 mL of media containing ethanol control or Nutlin-3a (final concentration, 8 μM) was added to each well. Photographs were taken with an Olympus IX50 inverted microscope at 4–40× magnification. To quantify the cell number, matrigel was digested using dispase (BD Biosciences) 0–96 h after initial plating. Media were removed from each well, and 2 mL of dispase was added per well and incubated at 37°C for 1 h. Each 3-mL sample was then transferred to a centrifuge tube, and 10 mM EDTA was added to stop the enzymatic activity of dispase. Each sample was centrifuged at 1000 rpm for 5 min and washed 3 times with PBS. Cells were then counted by trypan blue exclusion.

Real-Time Cell Analysis

Cell growth was monitored in real time using a Real-Time Cell Analyzer Dual-Plate instrument (Roche Applied Science) placed in a humidified incubator maintained with 5% CO2 at 37°C. This system records cell index (CI) as a unitless number that is a function of electrical impedance of cells attached to interdigitated electrodes built into the bottom of wells of a 16-well E-plate (Roche Applied Science). Recording of CI and subsequent data analysis were performed using the RTCA Software 1.2 (Roche Applied Science). Background impedance in each well in the presence of media alone was measured before cell seeding and automatically subtracted by the RTCA software. For proliferation assays, 5000–20,000 cells/well in 10% FBS containing DMEM or MEM media (Invitrogen) were seeded in E-plates. CI was recorded every 15 min for the next 96 h. Assays were performed at least twice with reproducible results.

Cell Cycle Analysis

Cells were plated at a density of 2.5 × 10^5 cells per well in 6-well plates in media containing 0.1% FBS. Cells were serum starved for 72 h to synchronize cells in G0. At 72 h, cells were harvested using trypsin, washed twice with DPBS + 10% FBS, fixed in ice-cold 80% ethanol, and stored at −20°C for at least 24 h. Remaining cells were treated with either ethanol or Nutlin-3a (final concentration, 8 μM) in media with 10% FBS. Treated cells were harvested and fixed at 24 h after the start of treatment. Fixed cells were incubated in 50 μL of PI buffer (20 μg/mL PI, 0.1% Triton-X 100, 200 μg/mL RNaseA in DPBS) for 30 min in the dark. Cells were then resuspended in 400 μL of DPBS for flow analysis. The samples were analyzed using a BD FACS Canto II cytometer (BD Biosciences) with BD FACS Diva software. All experiments were performed in triplicate and repeated at least twice.

Short Hairpin RNA Lentivirus Production and Infection

EGFP-tagged negative control and shWIP1 lentiviral expression constructs have been previously described. The HIV-EF-1-EGFP, psPAX2, and pVSVG plasmids were gifts from H. Trent Spencer (Emory University); 2 × 10^6 293T cells were plated on collagen-1–coated 100-mm dishes (BIOCAT; Becton Dickinson) and, 24 h later, were transfected with 8 μg EGFP-tagged empty vector (HIV-EF-1-EGFP) or the FG12-hv6-1-shWIP1 lentiviral vector along with 4 μg packaging construct, psPAX2, and 4 μg vesicular stomatitis virus G expression plasmid (pVSVG), using Lipofectamine 2000 (Invitrogen). Supernatant from transfected cells was collected at 48 and 72 h following...
transfection and stored at −80°C. Virus production was verified using the green fluorescent protein expression marker. Cell supernatant was pooled and centrifuged overnight at 10,000 × g at 4°C. Virus was resuspended in 1:200 volume serum-free DMEM media and stored at −80°C; 1 × 10^6 medulloblastoma cells were plated in 6-well plates and, 24 h later, were transduced with virus particles at a multiplicity of infection (MOI) of 2 with 8 μg/mL of polybrene. WIP1 knockdown following lentivirus infection was confirmed in cells by immunoblotting.

siRNA Transfection

For siRNA experiments, 1 × 10^6 medulloblastoma cells were plated in 6-well plates and, 24 h later, were transfected with 50 nM siWIP1, siHDM2, or negative control (siNC) siRNA oligomers (Dharmacon), using HiPerfect (Qiagen) reagent, according to the manufacturer's recommendations. Gene knockdown following siRNA transfection was confirmed at various time points by immunoblotting.

Dose-Response Viability Assays

D556 clones with stable expression of empty vectors (D556-pcDNA3), of WIP1 (D556-WIP1), and of a phosphatase-dead WIP1 (D556-WIP1 D314A) were either plated as adherent cell cultures or in matrigel and treated with 8 μM Nutlin-3a for 24–72 h, and then cell survival was determined by trypan blue exclusion. D556-pcDNA3 and D556-WIP1 cells were also plated as adherent cultures and treated with DMSO, 0.5 μM, or 5 μM CCT007093 (Sigma-Aldrich) for 6 days, and then cell survival was determined by trypan blue exclusion. Finally, D556-pcDNA3, D556-WIP1, and D425 cells were plated as adherent cultures and treated with ethanol, 8 μM Nutlin-3a, and shWIP1 lentivirus (MOI = 2), alone or in combination for 24–72 h, and then cell survival was determined by trypan blue exclusion. Experiments were done in triplicate and repeated at least twice.

Quantitative Real-Time RT-PCR

Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen). Total RNA (1 μg) was reverse transcribed into cDNA with Superscript III (Invitrogen) using random hexamers, as previously described. RNA integrity was verified using a Nanodrop spectrophotometer (ThermoScientific); only samples with an A260/A280 ratio more than 1.9 and less than 2.1 were used in quantitative real-time RT-PCR reactions. Quantitative real-time RT-PCR reactions containing cDNA, Syber Green PCR Master Mix (Applied Biosystems), and primers for human WIP1, HDM2, TATA-box Binding Protein (TBP), and/or Glyceraldehyde-3 Phosphate Dehydrogenase (GAPDH) in 25 μL final volume were performed for 45 cycles in triplicate on an ABI 7500 Real-Time PCR Cycler (Applied Biosystems) using absolute quantification with a standard curve. Serial dilutions of cDNA were used to determine a standard curve for each primer. Primer sequences are available upon request. Amplification products were verified by agarose gel electrophoresis and analysis of melting curves. WIP1 and HDM2 expression was normalized internally to TBP or GAPDH expression, accounting for differences in primer efficiencies. RNA expression was determined by the ΔΔCt method. Results from at least 3 separate experiments were analyzed.

Stereotactic Implantation of Tumor Cells

SCID/Beige mice (Charles River Laboratories) were anesthetized using 100 mg/kg ketamine (Pfizer) and 9 mg/kg xylazine (Ben Venue Laboratories) and were positioned in a stereotactic frame with a mouse adaptor (David Kopf Instruments). An incision was made in the midline of the scalp over the cerebellum, and a small hole was made in the skull (1 mm lateral to midline) using a bevelled (sharp point) 18G needle. A 24G Hamilton syringe loaded with 5 × 10^5 cells was mounted on a micromanipulator and introduced through the hole at a 30° angle to the surface of the cerebellum, at a depth of 1 mm. Cells in tissue culture media were injected over the course of 2 min, and the needle was left in place for another 2 min to avoid reflux. After removing the mouse from the frame, 1–2 drops of 0.25% (2.5 mg/mL) bupivicaine (Hospira) were applied along the incision for postoperative analgesia, and the skin was closed with 6-0 fast-absorbing plain gut suture using a 3/8 PC-1 cutting needle (Ethicon).

Mouse Handling

All mice were housed in an American Association of Laboratory Animal Care–accredited facility and were maintained in accordance with the National Institutes of Health guidelines. Mice were followed and sacrificed by CO₂ inhalation after they developed symptoms of medulloblastoma, which included head doming, hunched posture, preferential turning to one side, lethargy, and/or more than 15% weight loss. All animal care and experiments were approved by the Institutional Animal Care and Use Committee of Emory University (Protocol # 145-2009). Survival was analyzed using GraphPad Prism 4 software (GraphPad Software).

Mouse Necropsy and Tissue Handling

After euthanasia, the cranium and skull were removed from symptomatic mice using scissors and forceps. After severing the cranial nerves, we sagittally sectioned each mouse brain with a sterile scalpel and fixed them in 4% formalin for pathological examination. Tissue blocks were paraffin embedded, cut into 4-μm sections, and then stained with hematoxylin and eosin (H&E). Slides were processed in Emory’s Winship Cancer
Institute Histology Core and were visualized with an Olympus Provis AX-70 microscope (Olympus America). Images were captured with an Olympus DP70 digital camera and were analyzed using the DP Controller software package. Images were processed for publication using Adobe Photoshop Elements 5.0 (Adobe Systems).

H&E stained slides and immunohistochemistry for PCNA (Cell Signaling) of mid-sagittal sections from each mouse brain were examined by a pathologist (M.S.) using an Olympus CH-series light microscope. Three randomly selected, high-magnification fields (400 ×; field diameter, 0.45 mm) were assessed for numbers of mitotic figures on H&E slides and averaged for each specimen. Percent positivity of tumor cell nuclei for PCNA was determined by examination of a portion of one randomly selected 400 × field and counting positive and negative nuclei until a total of 200 cells were counted.

Statistical Analysis

Results were analyzed using a 2-tailed Student’s t test or 1-way analysis of variance (ANOVA) in Microsoft Excel or Graphpad Prism 4 software to assess statistical significance. Values of P < .05 were considered to be statistically significant.

Results

Group C and D Human Medulloblastomas Express WIP1 at High Levels

We have previously reported amplification or overexpression of WIP1 in 51% of human medulloblastomas. Another study has reported strong nuclear expression of WIP1 by immunohistochemistry in 148 (88%) of 168 human medulloblastomas on a tissue microarray. To place these prior findings in the context of recent reports that have categorized medulloblastomas into 3–5 distinct groups based on gene expression, we confirmed WIP1 expression in 2 independent medulloblastoma datasets. In the 62 human medulloblastomas profiled by Kool et al., WIP1 expression was 2.1-fold lower in group A’s WNT pathway-activated medulloblastomas, compared with the medulloblastomas in groups B–E (Fig. 1A). WIP1 expression was also 2-fold lower in combined groups A and Sonic-hedgehog (SHH) pathway-activated, group B, compared with medulloblastoma groups C–E (Fig. 1A). Conversely, WIP1 expression was 1.8-fold higher in the medulloblastomas in groups C and D combined than those in groups A, B, and E combined (Fig. 1A). WIP1 expression was also 1.6-fold higher in group D than other medulloblastoma groups (Fig. 1A).

Of the 103 independent medulloblastoma specimens profiled by Northcott et al., WIP1 expression was 1.3-fold lower in those activated by the SHH-pathway than in the other groups (Fig. 1B). Conversely, WIP1 expression was 1.8-fold higher in the medulloblastomas in group D than in the other groups (Fig. 1B). Northcott et al. also re-analyzed the Kool et al. dataset, separating those tumors into 4 rather than 5 categories. They report increased significance of the reduced expression of WIP1 in the medulloblastomas of the WNT-activated (P = 3 × 10⁻⁶) group and increased WIP1 expression in those of group D (P = 7.9 × 10⁻⁷) in the Kool et al. dataset. Because of the chromosomal locus of WIP1 on 17q22–23, these data are consistent with prior reports that have suggested that WNT- and SHH-activated medulloblastomas are biologically distinct from medulloblastomas with gain of chromosome 17q or i17q.

WIP1 Promotes Growth of TP53 Wild-Type Human Medulloblastoma Cell Lines

Because WIP1 is expressed at increased levels in group D and, possibly, in group C medulloblastomas, we next sought to understand the mechanisms by which WIP1 promotes medulloblastoma tumor growth. We first quantified WIP1 mRNA expression in human medulloblastoma cell lines. Because WIP1 over-expression or gene amplification has previously been described in human breast cancers that are wild-type for the TP53 gene, we examined WIP1 expression in the TP53 wild-type human medulloblastoma cell lines D425, Med8A, and D556 (http://www.sanger.ac.uk/genetics/CGP/CeLLines/) by quantitative real-time RT-PCR. We found that D556 cells express relatively low levels of WIP1 mRNA, comparable to levels previously described in the TP53 mutant Daoy cell line. D425 and Med8A cells expressed endogenous WIP1 mRNA at 13.7 and 5.6 times higher than in D556 cells, respectively (Fig. 2A).

Next, we generated stable, empty vector (pcDNA3), WIP1-expressing, and mutant, phosphatase-deficient WIP1-D314A clones of D556 cells and screened for expression of WIP1 transcript using quantitative real-time RT-PCR. D556 cells stably transfected with WIP1 (D556-WIP1) expressed up to 2.5 times more WIP1 mRNA than D556 cells with stable expression of an empty vector (D556-pcDNA; data not shown). D556 cells stably transfected with a mutant WIP expression vector (D556-WIP1 D314A) expressed WIP1 transcript by quantitative real-time RT-PCR that was 5–16 times higher than in D556-pcDNA stable cells (data not shown). Western blotting (Fig. 2B) revealed up to 15.5-fold higher WIP1 expression in D556-WIP1 clones and up to 194-fold higher WIP1 expression in D556-WIP1 D314A stable clones than in the D556-pcDNA clone 1B (Fig. 2B).

To assess the effect of increased WIP1 expression on medulloblastoma cell growth, D556-pcDNA, D556-WIP1, and D556-WIP1 D314A clones were plated at a density of 1 × 10⁴ cells/cm² and harvested at 0, 24, 48, and 72 h after initial plating. D556-WIP1 stable clones exhibited increased viability at all time points, compared with either D556-pcDNA or D556-WIP1 D314A stable clones (P < .0001, 1-way ANOVA) (Fig. 2C). We confirmed these findings by
examining anchorage-independent growth, a hallmark of transformed cells that refers to the ability of cells to proliferate in the absence of adhesion to a surface, which has been characterized as one of the best in vitro correlates of tumorigenicity.26 Seventy-two hours after initial plating in matrigel, cells were counted by trypan blue exclusion assays. D556-WIP1 cells demonstrated significantly increased viability, compared with D556-pcDNA or D556-WIP1 D314A stable-expressing clones ($P = .0001$, 1-way ANOVA) (Fig. 2D). We also plated 5000 D556-pcDNA, D556-WIP1, or D556-WIP1 D314A clones per well on E-plates and simultaneously monitored real-time changes in CI, which can be used as an indicator of cell proliferation, over 96 h.27,28 Log-phase growth of D556-WIP1 (clone 2-1F) cells was apparent as early as 12 h after plating and was apparent much earlier than in the D556-pcDNA or D556-WIP1 D314A clones (Supplementary material, Fig. S1A). This difference was quantified by measuring the slope of the CI and doubling times of all 3 cell lines 24–48 h after plating. The slope of the cell index of D556-WIP1 (clone 2-1F) cells was more than twice that of D556-pcDNA (clone 1B) and more than 12 times that of D556-WIP1 D314A (clone 1D) cells ($P = .0001$) (Supplementary material, Fig. S1B). The doubling time of D556-WIP1 (clone 2-1F) cells was 1.7 times faster than that of D556-pcDNA (clone 1B) cells and 5.3 times faster than that of D556-WIP1 D314A (clone 1D) cells ($P = .0022$) (Supplementary material, Fig. S1C).
WIP1 Does Not Alter the Growth of TP53 Mutant Daoy Medulloblastoma Cells

Having demonstrated the growth-promoting effects of over-expressed WIP1 in TP53 wild-type medulloblastoma cells, we hypothesized that mutation of TP53 would inhibit the proliferative effects of WIP1. Daoy medulloblastoma cells are known to contain a mutant TP53 gene (http://www.sanger.ac.uk/genetics/CGP/CellLines/). We have also previously shown that Daoy cells express low levels of WIP1 mRNA and protein.\textsuperscript{14}

To assess the effect of increased WIP1 expression in TP53 mutant medulloblastoma cells, we generated clones of Daoy cells that stably expressed empty vectors (pcDNA3), WIP1, and mutant, phosphatase-deficient WIP1-D314A clones of Daoy cells and then screened these clones for expression of the WPI protein. Western blotting (Fig. 3A and B) revealed up to a 7.1-fold higher WIP1 expression in Daoy-WPI clones and up to a 119-fold higher WIP1 expression in
Daoy-WIP1 D314A stable clones than in the Daoy-pcDNA clone 2C. To assess effects on proliferation, 1 Daoy-pcDNA, 3 Daoy-WIP1, and 2 Daoy-WIP1 D314A clones were plated at a density of $1 \times 10^4$ cells/cm$^2$ and harvested at 0, 24, 48, and 72 h after initial plating (Fig. 3C). At 24 and 48 h, there were no significant differences in viability among Daoy clones. At 72 h, there were fewer of both Daoy-WIP1 D314 clones 2D and 1A ($P = .0037$). However, there was no difference in viability between Daoy-pcDNA and Daoy-WIP1 stable clones.

**WIP1 Promotes Medulloblastoma Growth and Inhibits Mouse Survival In Vivo**

To validate the effects of WIP1 in vivo, we injected $5 \times 10^5$ D556-pcDNA (clone 1B) or D556-WIP1 (clone 2-1F) cells into the cerebellum of 3–6-month-old immunodeficient, SCID/Beige mice. Mice were observed daily and sacrificed after they exhibited symptoms of medulloblastoma, such as head doming, hunched posture, preferential turning to one side, lethargy, and/or significant weight loss. Mice bearing D556-WIP1 orthotopic xenografts ($n = 9$) exhibited inferior survival (median survival, 35 days following xenografting), compared with mice xenografted with D556-pcDNA cells ($n = 6$; median survival, 53 days; $P = .0002$) (Fig. 4A). The number of mice in each group differed because of problems with anesthesia (one death in each group of xenografts) or significant intracranial hemorrhage within a week of xenografting.

Nevertheless, a clear difference in both histology and proliferation could be seen in the D556-WIPI tumors. Compared with the D556-pcDNA xenografts, the D556-WIP1 xenografts were overall larger in size, had larger cells with more open chromatin and prominent nucleoli, and had more mitotic figures ($P = .0003$) (Fig. 4B). One of the D556-WIP1 xenografted animals even had distant intracranial metastases on the cerebral surface, detected by H&E staining. The D556-WIP1 xenografts had frequent pyknotic nuclear debris, which indicates a higher rate of apoptosis, although this debris was not noted in the D556-pcDNA xenografts. These findings are similar to the differences between classic and large-cell/anaplastic medulloblastoma in human clinical specimens. However, not all histological features of large-cell/anaplastic histology were noted in

![Fig. 3. Stable increased WIP1 expression failed to affect growth of TP53 mutant medulloblastoma cells. (A) Western blotting of whole cell lysates from TP53 mutant Daoy clones with stable expression of an empty vector (pcDNA), of WIP1, or of a mutated, phosphatase-deficient WIP1 (WIP1 D314A). (B) Scatter plot of quantified WIP1 expression, relative to β-actin and to expression in the Daoy empty-vector (Daoy-pcDNA) clone 2C. Error bars represent standard error of the mean. (C) Cell viability assayed by trypan blue exclusion in adherent cultures of Daoy clones 24–72 h following initial plating of cells (*$P = .004$ at 72 h). Error bars represent standard deviation among triplicates.](https://academic.oup.com/neuro-oncology/article-abstract/14/4/440/1053264)
the D556-WIP1 xenografts. We identified no unequivocal cell-cell wrapping and only modest nuclear molding. D556-WIP1 xenografted tumors did exhibit increased staining for the marker of proliferation, PCNA, compared with tumors in mice injected with the D556-pcDNA stable clone 1B ($P = .001$) (Fig. 4C), which likely explains the increased mortality of mice xenografted with D556-WIP1 cells.

WIP1-Driven Medulloblastoma Growth is Mediated by HDM2

HDM2 has previously been defined as an important target of WIP1’s phosphatase activity. WIP has been shown to dephosphorylate and, thus, stabilize HDM2, preventing it from ubiquitination and degradation through the 26S proteosome. We examined the expression of HDM2 in medulloblastoma cells by quantitative...
real-time RT-PCR and Western blotting. HDM2 mRNA expression was more than 2-fold higher in medulloblastoma cells with increased WIP1 expression, compared with the D556-pcDNA clone 1B (Fig. 5A). D556-WIP1 stable clones exhibited a slight increase in HDM2 protein expression, compared with clones containing empty vectors or mutant WIP1 D556 (Fig. 5A, right panel). Of interest, there was no clear difference in TP53 expression between WIP1 wild-type and mutant D556 clones.

To examine whether or not the effects of WIP1 on medulloblastoma growth were mediated by HDM2, we transfected medulloblastoma cells with either scrambled siRNA (siNC) or siRNA against HDM2 (siMDM2). The D556-WIP1 clone 2-1F exhibited reduced viability in response to HDM2 knockdown at
all time points examined (Fig. 5B). HDM2 knockdown did not affect the growth of D556-pcDNA cells. We next examined the effect of the HDM2 small molecule antagonist, Nutlin-3a, on medulloblastoma cell growth. Nutlin-3a prevents HDM2 from interacting with p53 and has been used to inhibit the growth of a variety of cancer cells in vitro, including Hodgkin’s lymphoma,30 acute lymphocytic leukemia,31 and neuroblastoma.32 Although Nutlin-3a inhibited anchorage-independent growth of D556-WIP1 stable cells by more than 50% (P < .0003), it did not affect the growth of D556-pcDNA or of mutant D556-WIP1 D314A clones (Fig. 5C and D). Similarly, while Nutlin-3a did not significantly alter the growth of D556-pcDNA cells grown as a monolayer (data not shown), it significantly inhibited the growth of adherent D556-WIP1 cells (P < .004) (Fig. 5E). We have shown that high WIP1 expression is associated with increased HDM2 expression. However, there are likely additional genetic differences among medulloblastoma models that affect HDM2 protein expression. Of importance, we showed that only D556 cells with increased expression of WIP1, compared with cells with stable expression of an empty vector or of mutant WIP1, exhibit sensitivity to genetic and pharmacologic inhibition of HDM2. This suggests that Nutlin treatment may be most effective in suppressing the growth of medulloblastomas that exhibit high WIP1 expression.

To confirm these findings, we synchronized D556 and Daoy stable clones in the G0 phase of the cell cycle by serum starvation for 72 h and then treated cells with either vehicle or Nutlin-3a for 24 h. Once released from G0 arrest, there was a significant shift of vehicle-treated D556-WIP1 (Clone 2-2B) cells into both the S phase (from 36% to 25%; P<.0001) (Fig. 8C). Together, our data suggests that Nutlin treatment may be most effective in suppressing the growth of medulloblastomas with high WIP1 expression.

Nutlin Potentiates the Effect of WIP1 Inhibition Against WIP1 High-Expressing Medulloblastoma Cells

Because inhibition of HDM2 and WIP1 independently suppressed medulloblastoma growth, we also examined the effect of combined inhibition of both targets. D556-WIP1 cells were treated with Nutlin-3a, shWIP1 lentivirus, or both (Fig. 9A). Seventy-two hours after the start of treatment with Nutlin or shWIP1, the viability of D556-WIP1 cells was reduced by 47.5% and 69.9%, respectively, relative to the vehicle-treated controls (P < .0007). Combined treatment with Nutlin-3a and shWIP1 resulted in an 80.9% reduction in viability determined by trypan blue exclusion, relative to the
Fig. 6. Nutlin-3 promotes the G0/G1 arrest of TP53 wild-type but not mutant TP53 medulloblastoma cells. Cell-cycle profiles of (A) TP53 wild-type D556 or (B) mutant TP53 Daoy medulloblastoma cell clones stably transfected with an empty vector (pcDNA), WIP1, or mutant WIP1 (D314A) arrested (time = 0) in G0 phase of the cell cycle by serum starvation. Cells were subsequently treated with serum-containing media and vehicle or Nutlin-3a for 24 h. Right panels, Mean percentages of cells in the G0/G1, the S, and the G2/M phases of the cell cycle. Error bars represent standard error among triplicates. Experiments were repeated at least twice.
Table 1. Nutlin-3 promotes the G0/G1 arrest of TP53 wild-type D556-WIP1 but not TP53 mutant Daoy-WIP1 medulloblastoma cells

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Discussion

Multiple studies have reported that gain of the long arm of chromosome 17 (17q) and isochoosome 17q (i17q), consisting of 17q deletion with duplication of 17q, are present in 30%–50% of medulloblastomas.4–6 Either loss of 17p (17p−) and/or gain of 17q have been associated with poor survival in patients who have received a diagnosis of medulloblastoma. Loss of the TP53 tumor suppressor gene, located on 17p13.1, and frequently deleted or mutated in 50% of adult cancers, has been considered a possible mechanism of tumorigenesis in 17p− medulloblastomas. However, prior studies have found that at least 50% of medulloblastomas with loss of heterozygosity of 17p maintain the TP53 gene locus.35 In addition, less than 10% of medulloblastomas have been reported to contain a TP53 mutation.36,37

The prevalence of the TP53 mutation and its significance in medulloblastoma biology have re-emerged as an area of controversy. A recent retrospective study reported 8 (16%) of 49 medulloblastomas had mutations in the DNA binding domain of TP53, and 19 (18%) of 108 showed strong staining for TP53 by immunohistochemistry. Strikingly, all TP53 mutated tumors were initially characterized as average-risk but exhibited local recurrence within 2 years after diagnosis and 0% 5-year survival, compared with 74% 5-year survival among patients bearing average-risk, TP53 wild-type medulloblastomas.38 Another study reports a 75% overall survival among patients whose medulloblastomas stain negatively, for TP53 compared with 35% overall survival among patients whose medulloblastomas stain positively. Three of 10 tumors that stained positively were found to harbor mutations of TP53.39

Neither evidence suggests that signaling through TP53 pathways is abnormal in medulloblastomas. Frank et al.41 described activation of the TP53-P14ARF pathway as a consequence of TP53 mutation, or homozygous deletion or methylation of the P14ARF promoter, particularly in large-cell/anaplastic medulloblastomas. Multiple transgenic mouse models...
have used Trp53 deletion to accelerate medulloblastoma tumorigenesis. We and others have described a role for splice variants of the TP53 family member TP73 in medulloblastoma formation. Amplification of the TP53 regulator HDM2 is another potential mechanism of medulloblastoma formation. Prior studies have reported that HDM2 amplification is present in 7% of human malignancies that lack mutation of p53. HDM2 over-expression has been associated with poor survival among adult patients with medulloblastoma; however, other groups have failed to document HDM2 amplification in pediatric medulloblastomas. We have described amplification and over-expression of the WIP1 oncogene in primary medulloblastomas and cell lines and have implicated high WIP1 expression in chemo-resistance. We have verified the results of previous studies that identified increased expression of PPM1D (i.e., WIP1) transcripts in a subset of medulloblastomas (Fig. 1). The infrequent nature of TP53 mutations in WIP1-amplified malignancies and the absence of TP53 mutation in most medulloblastomas suggest that increased WIP1 expression may be one mechanism by which gain of chromosome 17q promotes medulloblastoma growth. In this study, we investigated the role of the WIP1 oncogene on medulloblastoma growth in TP53 wild-

![Fig. 7. WIP1 knockdown inhibits growth of WIP1 high-expressing medulloblastoma cells. Representative photomicrographs of (A) D556-WIP1 (Clone 2-1F) and (D) D425 medulloblastoma cells 72 h following infection with enhanced green fluorescent protein (EGFP)-tagged shWIP1 or with empty vector (shNC)-containing lentiviral particles at a multiplicity of infection (MOI) of 2 (4×). Bars measure 200 µm. (B) Western blotting of whole cell lysates of shWIP1- and negative-control (NC)-infected D556-WIP1 (Clone 2-1F) cells. (C) Viability of D556-pcDNA (Clone 1B) and D556-WIP1 (Clone 2-1F) cells 0–72 h following infection with EGFP-empty vector or shWIP1 lentiviral particles at an MOI of 2, determined by trypan blue exclusion (*P < .0001). (D-E) Representative photomicrographs (magnification 4×, bars measure 200 µm) and viability of D425 medulloblastoma cells 0 to 72 h following infection with EGFP-shNC or EGFP-shWIP1 lentiviral particles at an MOI of 2, determined by trypan blue exclusion (*P < .0001). Error bars represent standard deviation among triplicates. All experiments were repeated at least three times.](https://academic.oup.com/neuro-oncology/article-abstract/14/4/440/1053264)
Fig. 8. CCT007093 inhibits growth of WIP1 high-expressing medulloblastoma cells. (A) Representative photomicrographs of D556-WIP1 (Clone 2-1F) cells 6 days after treatment with vehicle or with the WIP1 small molecule inhibitor CCT007093 (Mag. 4×). Bars measure 500 μm. (B) Viability of D556-WIP1 (Clone 2-1F) and D556-WIP1 D314A (Clone 1D) cells, determined by trypan blue exclusion, 6 days following treatment with vehicle or with 0.5–5 μM CCT007093 (*P < 0.005 at both doses). Error bars represent standard deviation among triplicates. (C) Western blotting of whole cell lysates of D556-WIP1 (Clone 2-1F) and D556-WIP1 D314A (Clone 1D) cells 72 h following treatment with vehicle or with 5 μM CCT007093. All experiments were repeated at least 3 times.

We verified the tumorigenic effects of WIP1 in vivo using human medulloblastoma cells and found that the level of expression of WIP1 significantly altered the survival of orthotopic, xenografted immunodeficient mice (Fig. 4). D556-WIP1 stable cells injected into the cerebellum of SCID/Beige mice reduced mouse survival by almost one-half to a median of 35 days, compared with survival of mice injected with the identical number of empty vector-transfected D556 cells. D556-WIP1 xenografted tumors exhibited some histological features of large-cell/anaplastic medulloblastoma, with increased atypia and mitotic figures and increased staining for a marker of cell proliferation, PCNA. Human large-cell medulloblastomas are a highly malignant variant that contain large, round, or pleomorphic nuclei with prominent nucleoli and more abundant cytoplasm than is found in non-large-cell medulloblastomas. Large-cell medulloblastomas also exhibit cell-cell wrapping, high mitotic activity, and a high apoptotic rate. Although D556-WIP1 xenografted murine medulloblastomas did not exhibit all the findings of human large-cell tumors, they were histologically distinct from tumors in mice orthotopically xenografted with empty vector D556 cells and were more aggressive with at least one D556-WIP1 xenograft that displayed evidence of leptomeningeal metastasis. Together, our in vitro and in vivo data suggest that WIP1 is an important tumor-promoting gene in medulloblastoma.
We further demonstrate that WIP1 knockdown is associated with increased phosphorylation of WIP1 targets and reduced growth of human medulloblastoma cells with stable (D556-WIP1) and endogenous high expression of WIP1 (D425) (Fig. 7). Treatment of WIP1-expressing medulloblastoma cells with the small-molecule WIP1 inhibitor CCT007093 similarly results in increased phosphorylation of the WIP1 target TP53 and dose-dependent growth inhibition of D556-WIP1 cells (Fig. 8). WIP1 knockdown had no effect on the growth of D556 empty vector-transfected cells. In addition, treatment with CCT007093 similarly had no significant effect on the growth of D556 cells containing a phosphatase-dead WIP1 gene. Our results suggest that medulloblastoma cells become addicted to the growth-promoting properties of the WIP1 gene and validate the potential use of a small molecule WIP1 antagonist as a targeted therapeutic agent in malignancies with high WIP1 expression.

The mechanism by which WIP1 promotes medulloblastoma growth is not entirely clear but is mediated in part by HDM2. D556-WIP1 and D556-WIP1 D314A cells both exhibited similar levels of expression and phosphorylation of p53. However, HDM2 levels

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**Fig. 9.** Combined inhibition of WIP1 and HDM2 enhances growth suppression of WIP1 high-expressing medulloblastoma cells. (A) Representative photomicrographs of EGFP-expressing D556-WIP1 (Clone 2-1F) cells 72 h after infection with enhanced green fluorescent protein (EGFP)-tagged shWIP1 or empty vector (shNC)-containing lentiviral particles at a multiplicity of infection (MOI) of 2 and treatment with either vehicle or 8 µM Nutlin-3a (Mag. 4×). Bars measure 500 µm. (B) Viability of D556-WIP1 (Clone 2-1F) cells treated as described in (A), assessed by trypan blue exclusion at 72 h (*P < .0007, both relative to EGFP-shNC infected and vehicle-treated cells; **P < .04 relative to either shWIP1-infected or Nutlin-3a-treated cells). Error bars represent standard deviation among triplicates. (C) Western blotting of whole cell lysates of D556-WIP1 (Clone 2-1F) cells treated as described in (A). (D) Representative photomicrographs of D556-WIP1 (Clone 2-1F) cells 6 days after treatment with vehicle, 4 µM Nutlin-3a, 5 µM CCT007093, or 4 µM Nutlin-3a along with 5 µM CCT007093 (Mag. 10×). Bars measure 500 µm. (E) Growth of D556-WIP1 (Clone 2-1F) and WIP1-mutated D556-WIP1 D314A (Clone 1D) cells treated as described in (D), assessed by trypan blue exclusion 6 days following treatment (*P < .005 relative to vehicle-treated controls; **P < .0002 relative to vehicle-treated controls; ***P < .03 relative to CCT007093-treated cells). Error bars represent standard deviation among triplicates. All experiments were repeated at least twice.
were slightly higher in D556-WIP1 stable clones (Fig. 5A). HDM2 knockdown inhibited growth of D556-WIP1 stable cells but did not affect the growth of D556 empty vector stable clones. Nutlin-3a, the HDM2 small molecule inhibitor, similarly and most significantly suppressed the growth of D556-WIP1 stable clones grown as adherent cells and in anchorage independence. We also provide evidence for increased growth suppression when both HDM2 and WIP1 are targeted in WIP1 high-expressing medulloblastoma cells (Fig. 9).

Recently published work suggests that aberrant HDM2 expression plays an important role in brain development and in medulloblastoma tumor formation. Mdm2, the mouse homologue of HDM2, is an E3 ubiquitin ligase that has been extensively characterized as an important inhibitor of the tumor suppressive function of p53. Absence of Mdm2 expression is known to result in death of mice in utero because of Trp53-mediated apoptosis; lethality can be fully rescued by deletion of the Trp53 gene. A recent study using mice that express a hypomorphic allele of Mdm2, Mdm2puro/Δ7-9 found that a 70% reduction in expression of Mdm2 protein results in hypoplasia and abnormal foliation of the developing cerebellum in mice. These findings were most pronounced in granule neuron precursor cells, a well-characterized cell of origin of de novo medulloblastoma tumors in mice. Mdm2puro/Δ7-9, Mdm2-deficient mouse granule neuron precursors exhibited increased expression of p53 and its downstream target genes, reduced proliferation, and increased apoptosis. The effects of Mdm2 expression on development of the cerebellum were highly dose-dependent, because mice with 50% Mdm2 expression did not exhibit noticeable cerebellar abnormalities. Furthermore, Mdm2 deficiency inhibited formation of preneoplastic lesions in medulloblastoma-prone Patched1-deficient (Pch1 +/−) mice. Mdm2 plays an important role not only in the appropriate development of the cerebellum but also through its effects on p53 function and therefore may be an important therapeutic target for the treatment of medulloblastoma.

Recent evidence also suggests that HDM2 does not function on its own in regulating the activity of TP53. A recent report suggests an important role for the U-box E3/E4 ligase ubiquitin factor E4B (UBE4B) in regulation of TP53 and in medulloblastoma tumorigenesis. U-box ubiquitin ligases feature a U-box domain, which is structurally related to the RING finger domain required for function of other ubiquitin ligases, such as HDM2. Mice harboring homozygous loss-of-function Ube4b mutations die in utero, whereas heterozygotes show multiple nervous system anomalies that worsen with age. UBE4B has recently been shown to be essential for HDM2-mediated polyubiquitination and degredation of TP53. The same study showed an inverse relation between TP53 expression and UBE4B amplification and over-expression in mouse medulloblastomas and in human cell lines and primary medulloblastomas, suggesting a novel target for treatment of pediatric brain tumors. It has yet to be determined whether UBE4B can be regulated by phosphorylation events and whether it may be a target of the WIP1 phosphatase.

In conclusion, we have demonstrated that the WIP1 phosphatase is important for medulloblastoma tumor growth in vitro and in vivo. Evidence in cell lines shows that high WIP1 expression is associated with increased expression of HDM2. Targeting HDM2 with Nutlin-3a phenocopies the effects of WIP knock-down or targeting with the small molecule drug CCT007093 in suppression of medulloblastoma growth. This suggests an important role for WIP1 and HDM2 inhibition, either alone or together, especially for the treatment of childhood medulloblastomas that exhibit high expression of the WIP1 oncogene.

**Supplementary Material**

Supplementary material is available at *Neuro-Oncology Journal* online (http://neuro-oncology.oxfordjournals.org/).

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