Published in final edited form as:

Int J Cancer. 2009 January 1; 124(1): 109–119. doi:10.1002/ijc.23929.

Defining a role for Sonic hedgehog pathway activation in desmoplastic medulloblastoma by identifying GLI1 target genes

Joon Won Yoon¹, Richard Gilbertson², Stephen Iannaccone¹, Philip Iannaccone¹, and David Walterhouse^{1,*}

¹Northwestern University Feinberg School of Medicine and the Developmental Biology Program of the Children's Memorial Research Center, Chicago, IL

²St. Jude Children's Research Hospital, Department of Developmental Neurobiology, Memphis, TN

Abstract

A subgroup of medulloblastomas shows constitutive activation of the Sonic hedgehog pathway with expression of *GLI1*. We identified the subset of GLI1 transforming target genes specifically expressed in medulloblastomas by comparing GLI1 targets in RK3E cells transformed by *GLI1* with the gene expression profile of Sonic hedgehog signature medulloblastomas. We identified 1,823 genes whose expression was altered more than 2-fold in 2 independent RK3E + *GLI1* cell lines. We identified 25 whose expression was altered similarly in medulloblastomas expressing *GLI1*. We identified potential GLI binding elements in the regulatory regions of 10 of these genes, confirmed that GLI1 binds the regulatory regions and activates transcription of select genes, and showed that GLI1 directly represses transcription of *Krox-20*. We identified upregulation of *CXCR4*, a chemokine receptor that plays roles in the proliferation and migration of granule cell neuron precursors during development, supporting the concept that reinitiation of developmental programs may contribute to medulloblastoma tumorigenesis. In addition, the targets suggest a pathway through which GLI1 may ultimately affect medulloblastoma cell proliferation, survival and genomic stability by converging on *p53*, *SGK1*, *MGMT* and *NTRK2*. We identify a *p53* mutation in RK3E + *GLI1* cells, suggesting that *p53* mutations may sometimes shift the balance toward dysregulated tumor cell survival.

Keywords

GLI1; medulloblastoma; *p53*; *CXCR4*; *SGK1*; *MGMT*; *NTRK2*

Medulloblastoma is the most common malignant brain tumor in childhood. Variants have been defined by histology (classic, desmoplastic, anaplastic and extremely nodular) and more recently by gene expression profiles.^{1–3} A subgroup that is characterized by upregulation of genes in the Sonic hedgehog (SHH) signal transduction pathway and is predominantly of desmoplastic histology has been identified that accounts for ~15–25% of medulloblastomas and may be associated with a more favorable prognosis.⁴ Mutations that lead to constitutive activation of the SHH pathway in medulloblastomas have been identified and usually target the SHH receptor, Patched 1 (*PTCH1*), or less frequently suppressor of

fused (*SUFU*) or smoothened (*SMOH*), leading to expression of the oncogenic transcription factor *GLI1*⁵⁻⁹ The role that upregulation of genes in the SHH pathway plays in the biology of this subgroup of medulloblastomas remains uncertain, although it has been suggested that aberrant activation of SHH signaling may reinitiate the developmental programs that control proliferation of external granule cells in the cerebellum and as a result be crucially involved in the tumorigenesis of desmoplastic medulloblastoma.¹⁰

The *Ptch*^{+/-} mouse model suggests a role for Shh pathway activation in the genesis of medulloblastoma.¹¹ However, since only a subset of these mice develop medulloblastoma, Shh pathway activation alone is presumably insufficient to induce the tumor and other initiating events must also occur. A dramatic increase in the incidence and accelerated development of medulloblastoma occurs in *Ptch*^{+/-} mice that also lack *p53*.¹² A high frequency of medulloblastoma formation has also been reported in mice following overexpression of *Shh* in cooperation with insulin-like growth factor signaling, *N-myc* and *Bcl2*.¹³⁻¹⁵ In other tumors, SHH pathway activation and *GLI1* expression have been associated with a range of functions, including tumor initiation, tumor progression and metastasis.¹⁶⁻²⁴

The expression of the transcription factor *GLI1* is dependent on SHH and therefore represents an accurate indicator of SHH pathway activity.^{25,26} As an oncogene, *GLI1* transforms rat kidney epithelial cells immortalized with adenovirus E1A (RK3E cells).²⁷ This model provides an advantage over studies of human tumor tissue of allowing identification of specific direct *GLI1* target genes following experimental manipulation of *GLI1* expression and has been used in gene expression profiling studies to identify target genes and pathways induced or repressed by *GLI1* during and following cell transformation.^{28,29} Unique sets of target genes have been identified in transformed RK3E cells for *GLI1* compared with other oncogenes, including *H-ras* and *c-MYC*. Limited overlap in *GLI1* targets was seen when the analysis was conducted during initiation of the transformed phenotype compared with following the establishment of the transformed phenotype in RK3E cells. However, a limited number of transcripts were evaluated in these studies decreasing the value of direct comparisons.

To identify roles that *GLI1* expression may play in medulloblastomas, we identified a subset of *GLI1* targets that are expressed in medulloblastomas by comparing *GLI1* target genes identified by gene expression profiles in RK3E cells transformed by *GLI1* (RK3E + *GLI1*) using oligonucleotide microarrays containing over 31,000 sequences with the gene expression profile that defines the subgroup of medulloblastomas defined by upregulation of the SHH pathway.³ We identify 25 overlapping genes, which collectively suggest that *GLI1* reinitiates a developmental program involving *CXCR4* that drives medulloblastoma cell proliferation and affects medulloblastoma cell proliferation, survival and genomic stability by converging on *p53*, serum/glucocorticoid regulated kinase 1 (*SGK1*), O-6-methylguanine-DNA methyltransferase (*MGMT*) and neurotrophic tyrosine kinase, receptor, type 2 (*NTRK2*). These effects may be modulated by *p53* mutations.

Material and methods

Preparation of cell lines

Rat kidney epithelial cells stably transfected with E1A (RK3E cells, American Type Culture Collection, CRL 1895) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µ/ml). Human *GLI1* in a retroviral vector (pLTR-*GLI1*) was introduced into RK3E cells by liposome-mediated transfection (RK3E + *GLI1*).

Transformed foci formed after 2 weeks in culture. Two cell lines were established by trypsinization of independent foci and called RK3E + *GLII*-I and RK3E + *GLII*-II.

Cell counting

RK3E and RK3E + *GLII* cells were counted using a hemocytometer following culture for up to 5 days. Triplicate counts were obtained and differences between cell types assessed using the Student's *t*-test.

Western blot analysis

Cell lysates were prepared using 1× lysis buffer (Promega, Madison, WI). Fifty to one hundred micrograms of total protein was separated using 7.5–12% SDS-PAGE and transferred to nitrocellulose membranes. GLII protein was detected using polyclonal anti-GLII antibody (Rockland, Gilbertsville, PA); p53 using a polyclonal anti-p53 antibody (Santa Cruz Biotech, Santa Cruz, CA); and β-actin using a monoclonal antibody (Sigma, St. Louis, MO). Antibodies were diluted 2,000- to 4,000-fold in PBS with 0.3% Tween-20. Protein was visualized using a chemiluminescence kit (Pierce, Rockford, IL).

Microarray assay

Total RNA was isolated from RK3E and RK3E + *GLII* cells using the Qiagen RNeasy RNA isolation kit (Qiagen, Valencia, CA). cDNA was synthesized using the SuperScript Choice system (Invitrogen, Carlsbad, CA). The manufacturer's protocol was modified slightly to include a high-performance liquid chromatography-purified T7-(dT) 24mer primer (Genset Corp., LaJolla, CA). Double-stranded cDNA was purified by phenol/chloroform extraction using phase lock gels (Eppendorf-5 Prime, Gaithersburg, MD) followed by ethanol precipitation. Biotin-labeled cRNA was prepared using the Enzo BioArray high yield RNA transcript labeling kit (Affymetrix, Santa Clara, CA). The *in vitro* transcription product was purified using RNeasy spin columns (Qiagen, Valencia, CA). We used oligonucleotide arrays (Affymetrix Rat 230 2.0) containing over 31,000 probe sets (Affymetrix, Santa Clara, CA). Hybridization, washing, staining and scanning were carried out using preprogrammed Affymetrix protocols. Data files (RK3E, RK3E + *GLII*-I and RK3E + *GLII*-II) were uploaded into Affymetrix Microarray Suite 5.0 (MAS5) software. Genes showing more than ±2-fold changes compared with RK3E cells were organized into a list. Genes that were upregulated in one cell line but downregulated in the other were excluded. The resulting list of genes was studied, and further information on genes of interest was compiled as tables. MIAME (minimum information about a microarray experiment) compliant data from our experiments has been deposited with GEO (Gene Expression Omnibus) at the National Center for Biotechnology Information (NCBI), GEO accession # GSE11987.

RT-PCR analysis

RT-PCR was performed using the Qiagen RT-PCR kit (Qiagen, Valencia, CA). cDNA was synthesized using random hexamers with 0.1 to 0.5 µg of total RNA. Identical reactions without RNA or without reverse transcriptase served as negative controls. cDNA was amplified using 32–35 PCR cycles. Amplified DNA was analyzed by agarose gel electrophoresis. Primers were designed using MacVector software (MacVector, Cary, NC). The primer sequences are shown, listing the forward primer (5'-3') followed by the reverse primer (5'-3'): Activated leukocyte cell adhesion molecule (*Alcam*) TTTTACTTACCAGGGCAGGCTG and CCTTCATCCACACCACAGTTG, chemokine (C-X-C motif) receptor 4 (*Cxcr4*) CAGAAGAAACCACACAGCACAACC and AAGGAAGTGAACGCTCCAGAATG, desmuslin (*Dmn*) GGAA GGCTAAGAAACATTTCAGTATC and TAGGTCTACTAAGGGC AGAGC, glycine amidinotransferase (*Gatm*) CCTTCAACATC ATTGGACCTGG and

TCATTGGCGTCTACCATCACG, *Mgmt* TCTCCATCACCTGTGTTCCAG and ATTGCTCCTCATCGC TCCTCCTAC, myosin IE (*Myo1E*) GCTGACTGATGGT CTTGCTTTCC and ATACTACCTGCGGCATTACATACG, *Ntrk2* GTCAAGTTTACCTAAGGGCACCTG and ATGGTTATCCGC ACTGGGAAG, *p53* TCTGTCATCCTTCCGTCCCTTCTC and GGCACAAACACGAACCTCAAAG, phosphoribosyl pyrophosphate synthetase-associated protein 1 (*Prpsap1*) AGACCAATGG AGAAACCAGAGTTG and GGGGAAGCAGAAAAAGCCTTG, phospholipid transfer protein (*Pltp*) CGGTTCTTGTCATCA CTTCTCG and AATGAGCGTATCTGGCGTGG, protein kinase C, eta (*Prkch*) GCAGGATGACGATGTGGAATG and GTCT CTGTAGATGATGCCTTTCTCG, solute carrier family 19, member 1 (*Slc19a1*) TGACCTTCGTGCTTTTCCGTG and GAAC TGTGATGGACTTGGAGGC, slit homolog 2 (*Slit2*) GCGGAT TATCTCCACACCAACC and TTTCAGGACAAGCCAAGT CTGC and *GAPDH* ATCACCATCTTCCAGGAGCG and CTAAGCAGTTGGTGGTGC. We performed multiple RT-PCRs using the same batch of RNA and always included a control *GAPDH* RT-PCR. The levels of *GAPDH* in RK3E and RK3E+ *GLI1* RNAs were comparable. Representative *GAPDH* RT-PCR bands for RK3E and RK3E+*GLI1* RNA are shown.

Identifying *GLI1* target genes in medulloblastomas

We compared the genes whose expression was altered more than ± 2 -fold in RK3E + *GLI1* cells to those that define each of the previously described 5 subgroups of medulloblastomas based on analysis using Affymetrix U133 av2 chips.³ Human orthologs of rat genes were obtained using NetAffx analysis (Affymetrix, Santa Clara, CA). Genes present in both rat and human arrays were selected using Excel software if their regulation was altered in the same direction (Microsoft corp., Redmond, WA). Genes whose regulation was in the opposite direction were excluded from this analysis.

Identification of potential direct targets of *GLI1*

Of the 25 genes whose expression was altered similarly in RK3E + *GLI1* cells and medulloblastomas with SHH pathway activation, 11 contained regulatory regions that had been previously characterized. We searched these regulatory regions for the GLI consensus binding motif, GACCACCCA, and for sequences with a mismatch in 1 or 2 loci using MacVector software (MacVector, Cary, NC). The regulatory regions of the remaining 14 genes had not yet been clearly defined and therefore were not analyzed for the GLI-specific DNA binding motif.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were performed as described previously.²⁸ Probes were designed using MacVector software (MacVector, Cary, NC). Probe sequences are shown, listing the sense sequence (5'-3') followed by the anti-sense sequence (5'-3') used to produce the double-stranded probe: NK6 transcription factor related, locus 1 (*hNKX6.1-1*) TGTAGTACTT GAC-CACCCACACCTCAATC and GATTGAGGTGTGGGTGGT CAAGTACTACA, *hNKX6.1-4* CCAGCTGCTGTGGGTGGAC TTCCAGAGGA and TCCTCTGGAAGTCCACCCACAGCAG CTGG, bone morphogenic protein-7 (*hBMP-7-1*) CAAACCA AAGGACCACCAAGGAGGGTGCA and TGCACCCTCCTTGG TGGTCCTTTGGTTTG, *hBMP-7-3* GGACTCCCCAGACCACT CAGAACCCACCG and CGGTGGGTTCTGAGTGGTCTGGGG AGTCC, *hPRPSAP1-1* GGCCTCTGGAGCCACACACTGCC CTATA and TATAGGGCAGTGTGTGGGCTCCAGAGGCC, *hPRPSAP1-2* GGGTAACACGGAGCACTCAGGAATACACG and CGTGTATTCCTGAGTGCTCCGTGTTACCC, *hPRPSAP1-3*

TCAGCGCCTTCCCCACCCAAACCGGGGTC and GACCC CGGTTTGGGTGGGGAAGGCGCTGA, *hPRPSAP1*-4 AAGG GGGCGGTGGGAGGGCCGGGCCGGGG and CCCC GGCCCTCCACCGCCCCCTT and *mKrox-20* CCACGCAGCG GACCGCCCAGACACGCCGG and CCGGCGTGTCTGGGCG GTCCGCTGCGTGG. The nonspecific probe sequences were GACTCTCCCGAAGAAGTGGGACAATGATGGTTC and GAC TGAACCATCATTGTCCCACTTCTTCGGGAG. The purified GLI1 protein used for electrophoretic mobility shift assays included aa 211–1106. The control protein was either PinPoint protein (Promega, Madison, WI) or PinPoint protein fused to GLI1 aa 879–1106, lacking the zinc finger DNA binding domain.

Preparation of reporter constructs that contain regulatory regions of GLI1 target genes

The human Cyclin D2-luciferase reporter was obtained from Milner (CV Therapeutics, Palo Alto, CA). The Cyclin D2-luciferase reporter was modified by inserting 3 tandem copies of the region of the Cyclin D2 promoter that contains the GLI binding site (5' - GACTTCTGCTCGCCACCAATCCTCGCT-3') into the MluI site to make the pGL3-hCyclin D2 reporter. Nine hundred and twenty-two base pairs of the mouse *Krox-20* promoter, including the region containing a GLI binding site (GACCGCCCA), were PCR amplified from genomic DNA (sense 5'-AAGGTGTGTTGAGTGGGTTTGTAAG-3' and anti-sense 5'-GCTCGCCAGGAACGATTTG-3') and inserted into the SalI/ XhoI site of the pGL3 basic vector to make an *mKrox-20*-luciferase reporter (pGL3-*mKrox-20*). A 2,041 base pair fragment of the human *PRPSAP1* gene, containing 4 potential GLI binding sites (GCCACACA, GAGCACTCA, CCCCACCCA, TGGGAGG GC) was PCR amplified (sense 5'-CTTCAGACTTGTGGTCCTT CAC-3' and anti-sense 5'-TTGAGAGTGGCGGCGGTCAGA TTC-3') and inserted into the TA cloning vector. *hPRPSAP1* DNA was retrieved by KpnI/EcoRV digestion and then ligated into the KpnI/ SmaI site of the pGL3 basic vector to make an *hPRPSAP1* reporter (pGL3-*hPRPSAP1*). One thousand five hundred and twelve base pairs of the regulatory sequence of the human *NKX6.1* gene, containing a GLI binding site (GACCA CCA), were PCR amplified (sense 5'-TCAATGCCTAACATCT ACCAGTCG-3' and anti-sense 5'-GCCTGAATGAGCGGTGAT TACAC-3') and ligated into the SalI site of the pGL2 promoter vector to produce the *hNKX6.1* reporter (pGL2-*hNKX6.1*).

Cotransfection assays

4×10^5 HeLa cells were grown in minimal essential medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. Cells were cotransfected using 6 μ l of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) with 0 to 1,000 ng of either the pM-*GLI1* or pCMV-*GLI1* effector plasmid, 600 ng of the reporter construct and 10 ng of Renilla control reporter DNA (Promega, Madison, WI). pM-*GLI1* and pCMV-*GLI1* were described previously.³⁰ pM (Clontech, Mountain View, CA) or pcDNA3 (Stratagene, LaJolla, CA) vectors served as control effectors. A total of 3 μ g of DNA was transfected in each experiment and the difference was made up with pBluescript carrier DNA (Stratagene, LaJolla, CA). Cell lysates were prepared 48 hr after transfection. One hundred microliters of substrate solution (Promega, Madison, WI) was added to 20 μ l of cell lysate. The experiments were performed at least in triplicate and results expressed as an average with standard deviation. Statistical significance was assessed using the Student's *t*-test.

Establishing interactions among GLI1-responsive genes

To identify functional interactions among GLI1 responsive genes, we used Pathway Architect Software (Stratagene, LaJolla, CA) and NCBI database searches. In particular, we used search modes including gene regulation, protein binding and protein-promoter binding.

We expanded the analysis to identify genes important for relating the GLI1 responsive genes using network expansion. Genes originally identified by Pathway Architect were crosschecked with literature searches and unrelated genes were eliminated from the network. A diagram was prepared using Graph Visualization software.

Transient transfection of RK3E cells with pCMV-GLI1

Ten micrograms of pCMV-*GLI1* or pcDNA3 were transfected into RK3E cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and OptiMEM (Gibco-BRL, Grand Island, NY). Cell lysates were prepared after 48 hr.

DNA sequence analysis

DNA sequencing was performed using the 3730xl DNA analyzer (Applied Biosystems, Foster City, CA).

Results

The gene expression profile that defines transformed RK3E + GLI1 cells

We established 2 independent cell lines from RK3E cells stably transfected with human *GLI1* (RK3E + *GLI1*-I and RK3E + *GLI1*-II) that had lost contact inhibition and formed foci in culture (Fig. 1a). The transformed cell lines showed strong GLI1 protein expression and accelerated cell proliferation compared with the parental cells (Figs. 1b and 1c). We used oligonucleotide arrays (Affymetrix Rat 230 2.0), containing over 31,000 probe sets to identify GLI1 targets in the RK3E + *GLI1* cell lines, characterized by this transformed phenotype; the phenotype we believed to be most comparable to an established human tumor.

We identified 1,823 genes whose levels of expression were altered ± 2 -fold in RK3E + *GLI1*-I and RK3E + *GLI1*-II cell lines compared with RK3E cells; 572 were upregulated and 1,251 downregulated (Supp. Info. data). We conducted RT-PCR to confirm changes in expression for select genes (Fig. 2). Of interest, the RK3E + *GLI1* gene expression profile includes coordinated regulation of several genes that are known to function in the Wnt signaling pathway, including downregulation of pathway activators (*Wnt2b*, *Wnt5b* and Frizzled homolog 1 [*Fzd1*]) and upregulation of negative regulators of the pathway (glypican3 and Wnt inhibitory factor 1 [*Wif1*]); the Bmp signaling pathway, including downregulation of *Bmp-1*, 2, 4, 6, and bone morphogenic protein receptor, type II (*Bmpr2*) and upregulation of *Bmp-7*; the Notch signaling pathway, including upregulation of Notch1, Notchless (*Nle1*), and downregulation of *Jag1* and *Adam10*; the chemokine pathway, including upregulation of *Cxcr4*, chemokine (C-X-C motif) ligand (*Cxcl*) 4, 9 and 10, and downregulation of *Cxcl12*; and the receptor tyrosine kinase (RTK) signaling pathway, including upregulation of *Stat2* and downregulation of *Ntrk2*.³¹⁻³⁹

Potential GLI1 targets in human medulloblastomas

To begin to define GLI1 targets in medulloblastomas and therefore the role of *GLI1* in medulloblastoma biology, we then compared expression of the GLI1 target genes identified in the RK3E cell transformation model with previously described gene expression profiles that define 5 subgroups of medulloblastomas.³ We selected genes whose expression patterns were identical in RK3E + *GLI1* cells and each of the medulloblastoma subsets. Seventy-one genes from those that define the subgroup of medulloblastomas with SHH pathway activation and predominantly of desmoplastic histology (called Subgroup D) were initially identified in this analysis.³ However, only 25 showed the same expression pattern compared to those identified in RK3E + *GLI1* cells (Table I). The other previously identified

medulloblastoma subgroups showed altered expression of very few of these 25 genes. Subgroups A and C did not show altered expression of any of the genes, while 4 in subgroup B (*MGMT*, *SGK1*, *PLTP* and *TST*) and 2 in subgroup E (*DNAJB9* and *KIDINS220*) showed comparable altered expression, confirming a close association of the list of 25 genes with the activation of the SHH signaling pathway and *GLI1* expression.³

Identification of direct targets of GLI1 in RK3E + GLI1 cells and medulloblastomas

To identify direct targets of GLI1, we searched the promoter and/or enhancer regions of select genes identified in the RK3E + *GLI1* cells for the GLI-specific DNA binding motif, GAC-CACCCA, and for sequences with a mismatch in 1 or 2 loci. We found potential consensus binding elements in the regulatory regions of 10 of the 25 genes (Table I). We analyzed the regulatory regions of 3 additional genes (*hBMP-7*, *hNKX6.1* and *mKrox-20*) whose expression was altered in RK3E + *GLI1* cells, but not medulloblastomas, based on their known roles in early development and the fact that their regulatory regions had been previously defined. We found potential consensus binding elements in each of them (Table II). We performed electrophoretic mobility shift assays to determine whether GLI1 physically binds select regulatory regions. The probes with putative GLI binding elements from *hBMP-7*, *hNKX6.1*, *mKrox-20*, and 2 sites in the 5' region of *hPRPSAP1* shifted in the presence of GLI1 protein, suggesting that these genes could represent direct targets (Fig. 3).

To determine whether GLI1 regulates transcription through these regions, we cotransfected HeLa cells with either the pM-*GLI1* or pCMV-*GLI1* expression vectors and luciferase reporters, containing the regulatory regions of Cyclin D2 (pGL3-hCyclin D2), *mKrox-20* (pGL3-*mKrox-20*), *hPRPSAP1* (pGL3-*hPRPSAP1*) or *hNKX6.1* (pGL2-*hNKX6.1*). We had previously identified a GLI consensus binding element in the regulatory region of human Cyclin D2.²⁸ The 150-kD GLI1 protein was not detected endogenously in HeLa cells (data not shown). As increasing amounts of the *GLI1* expression constructs were cotransfected with a constant amount of either the pGL3-hCyclin D2 or the pGL3-*hPRPSAP1* reporter construct, luciferase activity increased in a concentration dependent manner, suggesting that GLI1 directly activates transcription through these regulatory regions (Figs. 4a and 4b). Surprisingly, as an increasing amount of the *GLI1* expression construct was cotransfected with a constant amount of the pGL3-*mKrox-20* reporter construct, luciferase activity decreased to a maximum of 2-fold in a concentration dependent manner (Fig. 4c), suggesting that GLI1 directly represses transcription of *mKrox-20* through this regulatory region. Since it has generally been accepted that GLI1 functions only as a transcription activator and does not undergo cleavage, we looked for full length GLI1 in HeLa cells and RK3E cells using an anti-GLI1 polyclonal antibody prepared from a C-terminal GLI1 fragment. We identified full-length GLI1 protein in both cell lines (data not shown). This suggests that full-length GLI1 may indeed function as a transcription repressor at least under some circumstances for some promoters. By this method, GLI1 did not change reporter gene transcription through the *hNKX6.1* promoter region (Fig. 4d). Therefore, we were frequently able to provide evidence that GLI1 directly regulates transcription through regulatory regions of genes that contain GLI consensus elements, supporting the idea that the 10 genes that contain GLI consensus elements potentially represent direct targets.

Functional relationships among the GLI1-responsive genes in medulloblastoma

In an effort to identify functional relationships among the GLI1-responsive genes in medulloblastomas with SHH pathway activation, we created a regulatory network of the genes using the Direct Interaction Network algorithm of Pathway Architect software (Stratagene, LaJolla, CA) and verified the associations with the primary literature or NCBI databases (Fig. 5). The network appears to converge on genes that regulate cell proliferation, survival and genomic stability, including *p53*, *SGK1* and *MGMT*. In addition, c-MYC and

GLI1 activate several of the same targets, including *p53*, *PRPSAP1*, *RGS16* and *NPM1*; MAX and GLI1 regulate *PRPSAP1*, *RGS16* and *DNAJB9*; and TAF1 interacts with multiple GLI1 targets with diverse functions, including *PRPSAP1*, *NPM1*, *RGS16*, *DNAJB9* and *PDE4B*, suggesting that c-MYC, MAX and TAF1 function cooperatively with GLI1 during tumorigenesis.

Mutant p53 in RK3E + GLI1 cells

It is intriguing that microarray analyses demonstrate upregulation of *p53* in RK3E + *GLI1* cells and in medulloblastomas with SHH pathway activation. We confirmed this observation at the RNA level (Fig. 2). We also demonstrated upregulation of p53 protein following transfection of *GLI1* into RK3E cells (Fig. 6a). Since wild-type *p53* inhibits cell survival and the transformed phenotype, yet *GLI1* functions as an oncogene, we looked for *p53* mutations in RK3E + *GLI1* cells, as a possible explanation for GLI1-induced increases in p53 in transformed cells. We identified a *p53* point mutation (R246H) in the RK3E + *GLI1* cells (Fig. 6b). These data imply that p53 and GLI1 may work cooperatively to ensure normal cellular homeostasis, however, in the setting of *p53* mutations the balance may shift toward pathologic cell survival.

Discussion

We have established the gene expression profile of *GLI1* transformed RK3E cells by interrogating over 31,000 DNA sequences, representing the majority of the transcriptome. We identified 1,823 genes whose expression was altered more than 2-fold in RK3E + *GLI1* cells that were selected based on their transformed phenotype, which included loss of contact inhibition and an increased proliferative rate. We then used the RK3E + *GLI1* gene expression profile to identify roles that *GLI1* may play in medulloblastomas by comparing GLI1 target genes in RK3E + *GLI1* cells with the gene expression profile that defines the subgroup of medulloblastomas defined by upregulation of the SHH pathway. We identified 25 genes whose expression was altered similarly. These genes suggest roles for *GLI1* expression in reinitiating a developmental pathway that regulates medulloblastoma cell proliferation through CXCR4, and in regulating cell proliferation, survival and genomic stability by converging on *p53*, *SGK1*, *MGMT* and *NTRK2*. These effects may be modulated by *p53* mutations.

We observed coordinated regulation of genes involved in the Wnt, Bmp, Notch, chemokine and RTK signaling pathways in the RK3E + *GLI1* cells, suggesting crosstalk between the Hedgehog signal transduction pathway and each of these pathways in the transformed cells. Crosstalk with the Wnt, BMP and Notch pathways was not specifically identified when the RK3E + *GLI1* gene expression profile was compared with the profile that defines medulloblastomas with SHH pathway activation, suggesting that crosstalk between the signal transduction pathways is context specific and therefore highly regulated. It will ultimately be important to understand mechanisms regulating and modifying crosstalk between signal transduction pathways when developing, using and combining molecular targeted therapy for human cancers, including medulloblastomas.

In contrast, downregulation of *NTRK2*, an RTK signaling pathway member, was observed in the RK3E + *GLI1* cells and in medulloblastomas with SHH pathway activation. *NTRK2* is expressed in granule cell precursors in the external granule cell layer of the cerebellum and in 40–62% of medulloblastomas.^{40–42} In cultured cerebellar granule neurons, *NTRK2* initiated signal transduction enhances cell survival.^{40,43} While high levels of expression have been associated with a poor prognosis for neuroblastoma and other human cancers, there does not appear to be prognostic significance for *NTRK2* expression in medulloblastoma.^{44–46} The significance of downregulation in medulloblastoma biology

remains uncertain, although based on current knowledge likely affects cell survival programs. In addition, the chemokine receptor *CXCR4* was upregulated in the RK3E + *GLI1* cells and in the medulloblastomas with SHH pathway activation. While SHH is considered the major proliferative signal of cerebellar granule neuron precursors during development, the proliferative effect requires that CXCL12 binds CXCR4.⁴⁷ Indeed, *CXCR4* expression during development appears to not only play a direct role in the proliferation of granule cell precursors but also a role in retaining the cells in the proliferative environment of the external granule layer. *Cxcr4*^{-/-} mice suffer from severe cerebellar abnormalities, including a misplaced external granule layer and clusters of proliferating granule cell precursors that have migrated inappropriately.^{48,49} Upregulation of *CXCR4* has been previously correlated with the desmoplastic subtype of medulloblastoma and with *GLI1* expression using immunohistochemistry.⁵⁰ These results are confirmed by our study and also provide support for our method of identifying GLI1 targets in medulloblastoma. Indeed, upregulation of *CXCR4* by GLI1 suggests that GLI1 reinitiates a part of the developmental pathway in medulloblastomas that normally regulates proliferation of granule cell precursors.

One approach to understand the role of the transcription factor GLI1 in transformed cells and human cancers is to begin by identifying direct transcriptional targets. We established a paradigm to identify direct GLI1 targets genes, which includes, identification of the GLI consensus binding element, GACCACCCA, with up to 2 mismatches in the regulatory regions of a gene, demonstration that GLI1 binds the site by electrophoretic mobility shift assay and demonstration that GLI1 can regulate transcription through the region. We applied this approach to select genes and successfully demonstrated that GLI1 regulates transcription through most of the regulatory regions. However, we were not able to fully predict binding or activity based on the presence of a consensus sequence alone. For *PRPSAP1*, we demonstrated that GLI1 binds 2 of 4 consensus elements found in the regulatory region and regulates transcription through these sites. *PRPSAP1* is a component of phosphoribosylpyrophosphate synthetase complex, which catalyzes the production of 5' -phosphoribosyl 1-pyrophosphate (PRPP) from ATP and ribose 5'-phosphate.⁵¹ PRPP regulates *de novo* DNA synthesis during cell division. Roles in oncogenesis for *PRPSAP1* have not been previously described. Other genes, whose expression was altered in RK3E + *GLI1* cells and in medulloblastomas with SHH pathway activation, in which we identified consensus binding elements included *NTRK2*, *CXCR4*, *MGMT*, *SLC19A1*, *SLIT2*, *PRKCH*, *PLTP*, *MYCL1* and *CCPG1* and therefore become candidate direct GLI1 targets.

In contrast to GLI2 and GLI3, which function as transcription activators or repressors, it is generally accepted that GLI1 functions only as a transcription activator. GLI1 lacks a transcription repression domain found in the amino terminal regions of GLI2 and GLI3, and unlike GLI3, does not appear to undergo cleavage to a transcription repressor form.⁵² In our current array, 1,251 genes were downregulated following *GLI1*-induced cell transformation, presumably representing indirect targets. We identified GLI consensus binding elements in the promoter regions of some of the repressed genes, including *PRKCH*, *SLIT2*, *NTRK2* and *mKrox-20*. Surprisingly, we demonstrated that GLI1 binds the consensus binding element in the *mKrox-20* promoter and represses reporter gene transcription through this regulatory sequence in HeLa cells. Since Western analysis demonstrated the presence of full length GLI1 protein in this setting, it is possible that full length GLI1 functions as a transcription repressor, at least at certain promoters, under certain circumstances, which remain to be defined. We were unable to fully exclude the possibility of amino-terminal fragments of GLI1 if carboxy-terminal fragments were completely degraded.

We attempted to establish functional relationships among the subset of 25 genes that showed the same expression pattern in RK3E + *GLI1* cells and the subgroup of medulloblastomas with SHH pathway activation using pathway assist and NCBI literature searches. It is

possible that some of the remaining 46 genes that were regulated differently in the 2 systems still play important roles in the biology of medulloblastoma. Our analysis, which was validated by analyzing the specific published papers, lead to a pathway that appears to converge on genes involved in regulating cell proliferation, survival and genomic stability, including *p53*, *SGK1*, *MGMT* and *NTRK2*. Of interest, we identified *NTRK2*, *MGMT*, in addition to *CXCR4* as potential direct targets of *GLI1*.

SGK1 is a serine-threonine kinase in the AGC family that requires activation by the phosphatidylinositol 3' kinase signaling pathway.⁵³ Although functional roles appear to be cell type specific, Sgk1 has been shown to play roles in cell proliferation and survival in a variety of cell types, including mammary epithelial and tumor cells.^{54,55} Subcellular localization appears to be tightly regulated depending on the phase of the cell cycle in mammary epithelial cells, suggesting roles in cell cycle progression, and expression promotes survival of breast cancer cells through inactivation of the substrate FOXO3a.^{54,55} While increased expression has been described in some breast and hepatocellular carcinomas, downregulation of expression has been described in hormone-refractory prostate carcinoma.^{56–59} Precise roles within the context of cell proliferation and survival in these cancers remain incompletely understood. To the best of our knowledge, altered expression of *SGK1* in medulloblastoma has not been previously described and the functional significance of downregulation remains to be fully defined.

Loss of mismatch repair has been associated with genomic instability, which enables tumor initiation, progression and apoptosis. The DNA mismatch repair protein MGMT repairs O⁶-methylguanine, which restores DNA and promotes normal cell survival.⁶⁰ The expression of *MGMT* is inducible upon exposure to DNA damaging agents, including alkylating agents and ionizing radiation, and may require p53.^{61–63} It is expressed in highly variable amounts in human tumors, including medulloblastoma.⁶⁴ Higher levels of expression in medulloblastoma cell lines have been associated with resistance to alkylating agents, including temozolomide, BCNU and cyclophosphamide, which is commonly used in the treatment of medulloblastoma.^{65,66} However, MGMT upregulation does not appear to significantly enhance resistance to current therapy since patients with desmoplastic medulloblastomas have a more favorable outcome than patients with medulloblastomas that do not show activation of the SHH pathway. It is unclear whether MGMT contributes to the more favorable prognosis in this subgroup of medulloblastomas by protecting against genomic instability.

Perhaps, more surprising was the fact that *p53* was upregulated in transformed RK3E + *GLI1* cells and in medulloblastomas with SHH pathway activation. Indeed, *p53* overexpression has been observed in fewer than 10% of cases of medulloblastoma, and its prognostic significance remains unclear.^{67,68} Since loss of *p53* dramatically increased the incidence and accelerated development of medulloblastoma in *Ptch*^{+/-} mice,¹² and *p53* overexpression has been correlated with mutant forms of the protein, we looked for *p53* mutations in RK3E + *GLI1* cells. We identified a mutation affecting amino acid 246 (R246H), which is considered equivalent to amino acid 248 in human p53, a known mutational hot spot.⁶⁹ Of interest, the *GLI1*-expressing medulloblastoma cell line, Daoy, also carries a *p53* mutation (C242F).⁶⁸ Together, this suggests that at least sometimes, *p53* mutations may shift the balance toward dysregulated cell survival in *GLI1*-expressing medulloblastomas and that during development functional interactions between GLI1 and p53 may maintain normal cellular homeostasis.

Chromosome 17p deletions and isochromosome 17q (i17q), which combines loss of 17p and gain of 17q, are found in up to 50% of medulloblastomas and usually cause allelic deletion of *p53*.⁷⁰ However, it is estimated that only 12% of desmoplastic medulloblastomas

demonstrate these alterations⁷¹ and none of the tumors with SHH activation included in Subgroup D by Thompson *et al.* and used in our analysis, demonstrated 17p deletion or i17q.³ This suggests that other mechanisms of p53 inactivation would have to occur in the majority of desmoplastic medulloblastomas with SHH pathway activation. Since there is evidence that reduction in p53 levels as opposed to loss of both alleles may be sufficient to promote medulloblastoma tumor formation,⁷² other possible mechanisms of inactivating either one or both *p53* alleles could include point mutations, which have been described in ~10% of medulloblastomas,⁷³ or a previously undefined mechanism resulting from the unique gene expression profile in this subset of medulloblastomas. The lack of 17p deletions in the Subgroup D tumors may also in part explain why upregulation of p53 was even detected in this subset of tumors and in RK3E + *GLI1* cells, but not in the tumors included in Subgroup C by Thompson *et al.*, which were characterized by 17p losses/17q gains and sometimes activation of SHH pathway genes.³ Assessing the frequency of *p53* mutations in desmoplastic medulloblastomas may contribute to understanding tumorigenesis of this subset of medulloblastomas, which might be enhanced by *p53* mutations, and the more favorable response to therapy, which might be associated with upregulation of wild-type *p53*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant sponsor: Illinois Regenerative Medicine Institute initiative, the George M. Eisenberg Foundation for Charities, Illinois Department of Public Aid ("Excellence in Academic Medicine" Award).

References

1. Kleihues, P.; Cavenee, WK. Pathology and genetics of tumours of the nervous system. Lyon: IARC Press; 2000. International Agency for Research on Cancer.
2. Pomeroy SL, Tamayo P, Gaasenbeek M, Sturla LM, Angelo M, McLaughlin ME, Kim JY, Goumnerova LC, Black PM, Lau C, Allen JC, Zagzag D, et al. Prediction of central nervous system embryonal tumour outcome based on gene expression. *Nature*. 2002; 415:436–442. [PubMed: 11807556]
3. Thompson MC, Fuller C, Hogg TL, Dalton J, Finkelstein D, Lau CC, Chintagumpala M, Adesina A, Ashley DM, Kellie SJ, Taylor MD, Curran T, et al. Genomics identifies medulloblastoma subgroups that are enriched for specific genetic alterations. *J Clin Oncol*. 2006; 24:1924–1931. [PubMed: 16567768]
4. Rutkowski S, Bode U, Deinlein F, Ottensmeier H, Warmuth-Metz M, Soerensen N, Graf N, Emser A, Pietsch T, Wolff JE, Kortmann RD, Kuehl J. Treatment of early childhood medulloblastoma by postoperative chemotherapy alone. *N Engl J Med*. 2005; 352:978–986. [PubMed: 15758008]
5. Pietsch T, Waha A, Koch A, Kraus J, Albrecht S, Tonn J, Sorensen N, Berthold F, Henk B, Schmandt N, Wolf HK, von Deimling A, et al. Medulloblastomas of the desmoplastic variant carry mutations of the human homologue of *Drosophila* patched. *Cancer Res*. 1997; 57:2085–2088. [PubMed: 9187099]
6. Raffel C, Jenkins RB, Frederick L, Hebrink D, Alderete B, Fults DW, James CD. Sporadic medulloblastomas contain PTCH mutations. *Cancer Res*. 1997; 57:842–845. [PubMed: 9041183]
7. Reifenberger J, Wolter M, Weber RG, Megahed M, Ruzicka T, Lichter P, Reifenberger G. Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res*. 1998; 58:1798–1803. [PubMed: 9581815]
8. Taylor MD, Liu L, Raffel C, Hui CC, Mainprize TG, Zhang X, Agatep R, Chiappa S, Gao L, Lowrance A, Hao A, Goldstein AM, et al. Mutations in SUFU predispose to medulloblastoma. *Nat Genet*. 2002; 31:306–310. [PubMed: 12068298]

9. Wolter M, Reifemberger J, Sommer C, Ruzicka T, Reifemberger G. Mutations in the human homologue of the *Drosophila* segment polarity gene *patched* (PTCH) in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res.* 1997; 57:2581–2585. [PubMed: 9205058]
10. Pietsch T, Taylor MD, Rutka JT. Molecular pathogenesis of childhood brain tumors. *J Neurooncol.* 2004; 70:203–215. [PubMed: 15674478]
11. Wetmore C, Eberhart DE, Curran T. The normal *patched* allele is expressed in medulloblastomas from mice with heterozygous germ-line mutation of *patched*. *Cancer Res.* 2000; 60:2239–2246. [PubMed: 10786690]
12. Wetmore C, Eberhart DE, Curran T. Loss of p53 but not ARF accelerates medulloblastoma in mice heterozygous for *patched*. *Cancer Res.* 2001; 61:513–516. [PubMed: 11212243]
13. Rao G, Pedone CA, Del Valle L, Reiss K, Holland EC, Fults DW. Sonic hedgehog and insulin-like growth factor signaling synergize to induce medulloblastoma formation from nestin-expressing neural progenitors in mice. *Oncogene.* 2004; 23:6156–6162. [PubMed: 15195141]
14. Browd SR, Kenney AM, Gottfried ON, Yoon JW, Walterhouse D, Pedone CA, Fults DW. N-myc can substitute for insulin-like growth factor signaling in a mouse model of Sonic hedgehog-induced medulloblastoma. *Cancer Res.* 2006; 66:2666–2672. [PubMed: 16510586]
15. McCall TD, Pedone CA, Fults DW. Apoptosis suppression by somatic cell transfer of Bcl-2 promotes Sonic hedgehog-dependent medulloblastoma formation in mice. *Cancer Res.* 2007; 67:5179–5185. [PubMed: 17545597]
16. Oro AE, Higgins KM, Hu Z, Bonifas JM, Epstein EH Jr, Scott MP. Basal cell carcinomas in mice overexpressing Sonic hedgehog. *Science.* 1997; 276:817–821. [PubMed: 9115210]
17. Dahmane N, Lee J, Robins P, Heller P, Ruizi Altaba A. Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. *Nature.* 1997; 389:876–881. [PubMed: 9349822]
18. Hahn H, Wojnowski L, Zimmer AM, Hall J, Miller G, Zimmer A. Rhabdomyosarcomas and radiation hypersensitivity in a mouse model of Gorlin syndrome [see comments]. *Nat Med.* 1998; 4:619–622. [PubMed: 9585239]
19. Stein U, Eder C, Karsten U, Haensch W, Walther W, Schlag PM. GLI gene expression in bone and soft tissue sarcomas of adult patients correlates with tumor grade. *Cancer Res.* 1999; 59:1890–1895. [PubMed: 10213497]
20. Ghali L, Wong ST, Green J, Tidman N, Quinn AG. Gli1 protein is expressed in basal cell carcinomas, outer root sheath keratinocytes and a subpopulation of mesenchymal cells in normal human skin. *J Invest Dermatol.* 1999; 113:595–599. [PubMed: 10504446]
21. Aszterbaum M, Epstein J, Oro A, Douglas V, LeBoit PE, Scott MP, Epstein EH Jr. Ultraviolet and ionizing radiation enhance the growth of BCCs and trichoblastomas in *patched* heterozygous knockout mice. *Nat Med.* 1999; 5:1285–1291. [PubMed: 10545995]
22. Nilsson M, Unden AB, Krause D, Malmqwist U, Raza K, Zaphiropoulos PG, Toftgard R. Induction of basal cell carcinomas and trichoepitheliomas in mice overexpressing GLI-1. *Proc Natl Acad Sci USA.* 2000; 97:3438–3443. [PubMed: 10725363]
23. Karhadkar SS, Bova GS, Abdallah N, Dhara S, Gardner D, Maitra A, Isaacs JT, Berman DM, Beachy PA. Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature.* 2004; 431:707–712. [PubMed: 15361885]
24. Sheng T, Li C, Zhang X, Chi S, He N, Chen K, McCormick F, Gatalica Z, Xie J. Activation of the hedgehog pathway in advanced prostate cancer. *Mol Cancer.* 2004; 3:29. [PubMed: 15482598]
25. Lee J, Platt KA, Censullo P, Ruizi Altaba A. Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. *Development.* 1997; 124:2537–2552. [PubMed: 9216996]
26. Goodrich LV, Scott MP. Hedgehog and *patched* in neural development and disease. *Neuron.* 1998; 21:1243–1257. [PubMed: 9883719]
27. Ruppert JM, Vogelstein B, Kinzler KW. The zinc finger protein GLI transforms primary cells in cooperation with adenovirus E1A. *Mol Cell Biol.* 1991; 11:1724–1728. [PubMed: 1825351]
28. Yoon JW, Kita Y, Frank DJ, Majewski RR, Konicek BA, Nobrega MA, Jacob H, Walterhouse D, Iannaccone P. Gene expression profiling leads to identification of GLI1-binding elements in target

- genes and a role for multiple downstream pathways in GLI1-induced cell transformation. *J Biol Chem.* 2002; 277:5548–55. [PubMed: 11719506]
29. Louro ID, Bailey EC, Li X, South LS, McKie-Bell PR, Yoder BK, Huang CC, Johnson MR, Hill AE, Johnson RL, Ruppert JM. Comparative gene expression profile analysis of GLI and c-MYC in an epithelial model of malignant transformation. *Cancer Res.* 2002; 62:5867–5873. [PubMed: 12384550]
 30. Yoon JW, Liu CZ, Yang JT, Swart R, Iannaccone P, Walterhouse D. GLI activates transcription through a herpes simplex viral protein 16-like activation domain. *J Biol Chem.* 1998; 273:3496–3501. [PubMed: 9452474]
 31. Xu L, Corcoran RB, Welsh JW, Pennica D, Levine AJ. WISP-1 is a Wnt-1- and beta-catenin-responsive oncogene. *Genes Dev.* 2000; 14:585–595. [PubMed: 10716946]
 32. Kawai S, Sugiura T. Characterization of human bone morphogenetic protein (BMP)-4 and -7 gene promoters: activation of BMP promoters by Gli, a Sonic hedgehog mediator. *Bone.* 2001; 29:54–61. [PubMed: 11472891]
 33. You Z, Saims D, Chen S, Zhang Z, Guttridge DC, Guan KL, Mac-Dougald OA, Brown AM, Evan G, Kitajewski J, Wang CY. Wnt signaling promotes oncogenic transformation by inhibiting c-Myc-induced apoptosis. *J Cell Biol.* 2002; 157:429–440. [PubMed: 11980918]
 34. Cebrat M, Strzadala L, Kisielow P. Wnt inhibitory factor-1: a candidate for a new player in tumorigenesis of intestinal epithelial cells. *Cancer Lett.* 2004; 206:107–113. [PubMed: 15019166]
 35. Balkwill F. The significance of cancer cell expression of the chemokine receptor CXCR4. *Semin Cancer Biol.* 2004; 14:171–179. [PubMed: 15246052]
 36. Song HH, Shi W, Xiang YY, Filmus J. The loss of glypican-3 induces alterations in Wnt signaling. *J Biol Chem.* 2005; 280:2116–2125. [PubMed: 15537637]
 37. Capurro MI, Xiang YY, Lobe C, Filmus J. Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. *Cancer Res.* 2005; 65:6245–6254. [PubMed: 16024626]
 38. Langenfeld EM, Bojnowski J, Perone J, Langenfeld J. Expression of bone morphogenetic proteins in human lung carcinomas. *Ann Thorac Surg.* 2005; 80:1028–1032. [PubMed: 16122479]
 39. Ratajczak MZ, Zuba-Surma E, Kucia M, Reza R, Wojakowski W, Ratajczak J. The pleiotropic effects of the SDF-1-CXCR4 axis in organogenesis, regeneration and tumorigenesis. *Leukemia.* 2006; 20:1915–1924. [PubMed: 16900209]
 40. Zirngiebel U, Ohga Y, Carter B, Berninger B, Inagaki N, Thoenen H, Lindholm D. Characterization of TrkB receptor-mediated signaling pathways in rat cerebellar granule neurons: involvement of protein kinase C in neuronal survival. *J Neurochem.* 1995; 65:2241–2250. [PubMed: 7595513]
 41. Washiyama K, Muragaki Y, Rorke LB, Lee VM, Feinstein SC, Radeke MJ, Blumberg D, Kaplan DR, Trojanowski JQ. Neurotrophin and neurotrophin receptor proteins in medulloblastomas and other primitive neuroectodermal tumors of the pediatric central nervous system. *Am J Pathol.* 1996; 148:929–940. [PubMed: 8774147]
 42. Tajima Y, Molina RP Jr, Rorke LB, Kaplan DR, Radeke M, Feinstein SC, Lee VM, Trojanowski JQ. Neurotrophins and neuronal versus glial differentiation in medulloblastomas and other pediatric brain tumors. *Acta Neuropathol.* 1998; 95:325–332. [PubMed: 9560008]
 43. Nonomura T, Kubo T, Oka T, Shimoke K, Yamada M, Enokido Y, Hatanaka H. Signaling pathways and survival effects of BDNF and NT-3 on cultured cerebellar granule cells. *Brain Res.* 1996; 97:42–50.
 44. Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM. Expression and function of TRK-B and BDNF in human neuroblastomas. *Mol Cell Biol.* 1994; 14:759–767. [PubMed: 8264643]
 45. Segal RA, Goumnerova LC, Kwon YK, Stiles CD, Pomeroy SL. Expression of the neurotrophin receptor TrkC is linked to a favorable outcome in medulloblastoma. *Proc Natl Acad Sci USA.* 1994; 91:12867–12871. [PubMed: 7809137]
 46. Desmet CJ, Peeper DS. The neurotrophic receptor TrkB: a drug target in anti-cancer therapy? *Cell Mol Life Sci.* 2006; 63:755–759. [PubMed: 16568244]

47. Klein RS, Rubin JB, Gibson HD, DeHaan EN, Alvarez-Hernandez X, Segal RA, Luster AD. SDF-1 alpha induces chemotaxis and enhances Sonic hedgehog-induced proliferation of cerebellar granule cells. *Development*. 2001; 128:1971–1981. [PubMed: 11493520]
48. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature*. 1998; 393:595–599. [PubMed: 9634238]
49. Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT, Springer TA. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci USA*. 1998; 95:9448–9453. [PubMed: 9689100]
50. Schuller U, Koch A, Hartmann W, Garre ML, Goodyer CG, Cama A, Sorensen N, Wiestler OD, Pietsch T. Subtype-specific expression and genetic alterations of the chemokine receptor gene CXCR4 in medulloblastomas. *Int J Cancer*. 2005; 117:82–89. [PubMed: 15880586]
51. Tatibana M, Kita K, Taira M, Ishijima S, Sonoda T, Ishizuka T, Iizasa T, Ahmad I. Mammalian phosphoribosyl-pyrophosphate synthetase. *Adv Enzyme Regul*. 1995; 35:229–249. [PubMed: 7572345]
52. Dai P, Akimaru H, Tanaka Y, Maekawa T, Nakafuku M, Ishii S. Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. *J Biol Chem*. 1999; 274:8143–8152. [PubMed: 10075717]
53. Tessier M, Woodgett JR. Serum and glucocorticoid-regulated protein kinases: variations on a theme. *J Cell Biochem*. 2006; 98:1391–1407. [PubMed: 16619268]
54. Wu W, Zou M, Brickley DR, Pew T, Conzen SD. Glucocorticoid receptor activation signals through forkhead transcription factor 3a in breast cancer cells. *Mol Endocrinol*. 2006; 20:2304–2314. [PubMed: 16690749]
55. Buse P, Tran SH, Luther E, Phu PT, Aponte GW, Firestone GL. Cell cycle and hormonal control of nuclear-cytoplasmic localization of the serum- and glucocorticoid-inducible protein kinase, Sgk, in mammary tumor cells. A novel convergence point of anti-proliferative and proliferative cell signaling pathways. *J Biol Chem*. 1999; 274:7253–7263. [PubMed: 10066787]
56. Adeyinka A, Emberley E, Niu Y, Snell L, Murphy LC, Sowter H, Wykoff CC, Harris AL, Watson PH. Analysis of gene expression in ductal carcinoma in situ of the breast. *Clin Cancer Res*. 2002; 8:3788–3795. [PubMed: 12473591]
57. Chung EJ, Sung YK, Farooq M, Kim Y, Im S, Tak WY, Hwang YJ, Kim YI, Han HS, Kim JC, Kim MK. Gene expression profile analysis in human hepatocellular carcinoma by cDNA microarray. *Mol Cells*. 2002; 14:382–387. [PubMed: 12521301]
58. Sahoo S, Brickley DR, Kocherginsky M, Conzen SD. Coordinate expression of the PI3-kinase downstream effectors serum and glucocorticoid-induced kinase (SGK-1) and Akt-1 in human breast cancer. *Eur J Cancer*. 2005; 41:2754–2759. [PubMed: 16246546]
59. Zhang L, Cui R, Cheng X, Du J. Antiapoptotic effect of serum and glucocorticoid-inducible protein kinase is mediated by novel mechanism activating I{kappa}B kinase. *Cancer Res*. 2005; 65:457–464. [PubMed: 15695387]
60. Kaina B, Christmann M, Naumann S, Roos WP. MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. *DNA Repair*. 2007; 6:1079–1099. [PubMed: 17485253]
61. Fritz G, Tano K, Mitra S, Kaina B. Inducibility of the DNA repair gene encoding O6-methylguanine-DNA methyltransferase in mammalian cells by DNA-damaging treatments. *Mol Cell Biol*. 1991; 11:4660–4668. [PubMed: 1875945]
62. Rafferty JA, Clarke AR, Sellappan D, Koref MS, Frayling IM, Margison GP. Induction of murine O6-alkylguanine-DNA-alkyltransferase in response to ionising radiation is p53 gene dose dependent. *Onco-gene*. 1996; 12:693–697.
63. Grombacher T, Eichhorn U, Kaina B. p53 is involved in regulation of the DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT) by DNA damaging agents. *Oncogene*. 1998; 17:845–851. [PubMed: 9780001]
64. Bobola MS, Berger MS, Ellenbogen RG, Roberts TS, Geyer JR, Silber JR. O6-Methylguanine-DNA methyltransferase in pediatric primary brain tumors: relation to patient and tumor characteristics. *Clin Cancer Res*. 2001; 7:613–619. [PubMed: 11297257]

65. Bobola MS, Silber JR, Ellenbogen RG, Geyer JR, Blank A, Goff RD. O6-methylguanine-DNA methyltransferase, O6-benzylguanine, and resistance to clinical alkylators in pediatric primary brain tumor cell lines. *Clin Cancer Res*. 2005; 11:2747–2755. [PubMed: 15814657]
66. Friedman HS, Pegg AE, Johnson SP, Loktionova NA, Dolan ME, Modrich P, Moschel RC, Struck R, Brent TP, Ludeman S, Bullock N, Kilborn C, et al. Modulation of cyclophosphamide activity by O6-alkylguanine-DNA alkyltransferase. *Cancer Chemother Pharmacol*. 1999; 43:80–85. [PubMed: 9923545]
67. Miralbell R, Tolnay M, Bieri S, Probst A, Sappino AP, Berchtold W, Pepper MS, Pizzolato G. Pediatric medulloblastoma: prognostic value of p53, bcl-2, Mib-1, and microvessel density. *J Neurooncol*. 1999; 45:103–110. [PubMed: 10778725]
68. Raffel C, Thomas GA, Tishler DM, Lassofoff S, Allen JC. Absence of p53 mutations in childhood central nervous system primitive neuroectodermal tumors. *Neurosurgery*. 1993; 33:301–305. discussion 305–6. [PubMed: 8396224]
69. Mancuso T, Aguilar F, Pescarolo MP, Clerico L, Russo P, Parodi S. Mutation frequencies at codon 248 of the p53 tumour suppressor gene are not increased in colon cancer cell lines with the RER1 phenotype. *Nucl Acids Res*. 1997; 25:3643–3648. [PubMed: 9278485]
70. Griffin CA, Hawkins AL, Packer RJ, Rorke LB, Emanuel BS. Chromosome abnormalities in pediatric brain tumors. *Cancer Res*. 1988; 48:175–180. [PubMed: 3334992]
71. Gilbertson RJ, Ellison DW. The origins of medulloblastoma subtypes. *Ann Rev Pathol*. 2008; 3:341–365. [PubMed: 18039127]
72. Venkatachalam S, Shi YP, Jones SN, Vogel H, Bradley A, Pinkel D, Donehower LA. Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. *EMBO J*. 1998; 17:4657–4667. [PubMed: 9707425]
73. Ellison D. Classifying the medulloblastoma: insights from morphology and molecular genetics. *Neuropathol Appl Neurobiol*. 2002; 28:257–282. [PubMed: 12175339]

Figure 1.

Comparison of RK3E and RK3E +GLI1 cells. (a) Growth characteristics of the parental RK3E cells compared with 2 independent RK3E +GLI1 clones (RK3E +GLI1-I and RK3E +GLI1-II). (b) (Top) GLI1 expression was not detected by anti-GLI1 antibody in RK3E (parental cell line) cell lysate (lane 1) or in cell lysate from RK3E cells transformed by *H-ras* (lane 3). Stable GLI1 expression was detected by anti-GLI1 antibody in RK3E +GLI1 cell lysate (lane 2). Arrow indicates the GLI1 protein at ~150 kD. (Bottom) Cell lysates from RK3E +GLI1-I cells (lane 3) and RK3E +GLI1-II cells (lane 2) demonstrated GLI1 expression by anti-GLI1 antibody. Lysate from GLI1-amplified OsA cells serves as a positive control (lane 1). Arrow indicates the GLI1 protein at ~150 kD. (c) RK3E +GLI1 cells showed accelerated proliferation compared with RK3E cells. Asterisk (*) indicates statistical significance at day 2 ($p = 0.04$).

Figure 2. Confirmation of expression changes detected by microarray analysis using RT-PCR. Left column, RT-PCR analysis of select genes found to be induced in RK3E + *GLI1* cells compared with RK3E cells by microarray analysis. Right column, RT-PCR analysis of select genes found to be repressed in RK3E + *GLI1* cells compared with RK3E cells by microarray analysis. *Gapdh* is the reference gene.

Figure 3.

Electrophoretic mobility shift assays show that GLI1 binds regulatory regions that contain GLI consensus binding elements in select genes. The gene name and the sense strand of the GLI consensus binding element portion for each probe are shown above the corresponding gel. Each experiment was set up as, lane 1—probe alone, lane 2—probe + control protein, lane 3—probe + GLI1 protein, lane 4—probe + GLI1 protein + 100-fold molar excess of nonradiolabeled gene specific oligonucleotide and lane 5—probe + GLI1 protein + 100-fold molar excess of nonspecific oligonucleotide. Specific band shifts (arrows) that competed off with the specific competitor but not with the nonspecific competitor were demonstrated for *hBMP-7* (GACCACCCaA), *mKrox-20* (GACCgCCCA), *hNKX6.1* (GACCACCCA), *hPRPSAP1* (GcCCA-CaCA) and *hPRPSAP1* (GcGGtCCCA).

Figure 4.

Cotransfection assays in HeLa cells demonstrate that GLI1 regulates luciferase activity through the 5' regions of select genes. (a) HeLa cells were cotransfected with either the control pcDNA3 (gray bars) or pCMV-*GLI1* (black bars) vectors and the pGL3-hCyclin D2 reporter. Error bars are shown. Asterisks indicate statistically significant increases in luciferase activity between a given amount of pCMV-*GLI1* effector and no effector ($p < 0.01$). (b) Similarly, for *hPRPSAP1*, using the pM-*GLI1* effector and pGL3-*hPRPSAP1* reporter. Asterisks indicate statistically significant increases in luciferase activity between a given amount of pM-*GLI1* effector and no effector ($p < 0.01$) and between a given amount

of pM-*GLII* and pM control ($p < 0.01$). (c) For *mKrox-20*, using either the control pM (gray bars) or pM-*GLII* (black bars) and the pGL3-*mKrox20* reporter, asterisks indicate statistically significant repression of luciferase activity between a given amount of effector and no effector (0.125 μ g, $p = 0.04$; otherwise $p > 0.01$) and between a given amount of pM-*GLII* and pM control ($p < 0.01$). (d) For *hNKX6.1*, the experiment was conducted similarly, using the pGL2-*hNKX6.1* reporter. No changes in luciferase activity were detected.

Figure 5.

The GLI1 target genes converge on a regulatory pathway that suggests roles in cell proliferation, survival and genomic stability. Genes in yellow represent GLI1 targets identified by our analysis. Genes in blue, identified in the literature, are included based on a connection to the experimentally recognized genes. Dotted lines connect GLI1 targets, whether direct or indirect, identified here experimentally. Solid lines represent connections between genes identified in the literature. Green lines represent genes that are activated. Red lines represent genes that are repressed. Blue lines represent genes that are both activated and repressed. The following genes, shown here to be altered by GLI1, have not been previously published as controlled directly or indirectly by GLI1: *PLTP*, *GATM*, *PRKCH*, *TST*, *SLIT2*, *SLC19A1*, *KIDINS220*, *DMN*, *ANKRD15 myosin IE*, *CXCR4*, *MYCL1*. Gene abbreviations: *E2F4*, E2F transcription factor 4; *E2F1*, E2F transcription factor 1; *TAF1*,

TATA-binding protein associated factor 1; *PLCG1*, phospholipase C, gamma-1; *CSNK2A1*, casein kinase 2, alpha 1; *FYN*, FYN oncogene related to SRC; *GRB2*, growth factor receptor-bound protein 2; *PRNP*, prion protein; *AP1*, adaptor-related protein 1; *Bcl2*, B-cell CLL/lymphoma 2; *CREB1*, cAMP responsive element binding protein 1; *CBP*, CREB binding protein; *MAX*, Myc associated factor X; *Myc*, v-myc myelocytomatosis viral oncogene homologue (avian); *P300*, E1A binding protein p300; *SGK1*, serum/glucocorticoid regulated kinase 1; *HNF4A*, Hepatocyte nuclear factor 4, alpha; *YY1*, YY1 transcription factor; *P16/INK4*, cyclin-dependent kinase inhibitor 2A. The remaining gene abbreviations are defined in Table I.

Figure 6.

Expression of a mutant form of *p53* increases in RK3E cells following transient transfection with *GLI1*. (a) Transient transfection of RK3E cells with pCMV-*GLI1* results in increased *p53* expression by Western blot analysis. Lane 1—*p53* expression in RK3E cells, lane 2—*p53* expression in RK3E cells transfected with the control pcDNA3 vector that does not express *GLI1* and lane 3—*p53* expression in RK3E cells transfected with pCMV-*GLI1*. The membrane was stripped and reprobed with β -actin antibody to ensure equal loading. (b) DNA sequence analysis of endogenous *p53* in RK3E cells demonstrates a point mutation (R246H).

TABLE I

Genes that demonstrate altered expression in RK3E + *GLI1* cells and medulloblastomas with SHH pathway activation and analysis of their promoter regions for GLI consensus binding elements

Gene abbreviation	Gene name	Expression	Promoter	GLI consensus binding sites
<i>ALCAM</i>	Activated leukocyte cell adhesion molecule	↓	–	
<i>ANKRD15</i>	Ankyrin repeat domain 15	↓	–	
<i>Ccp1</i>	Cell cycle progression 1 (predicted)	↓	+	cACctCCCA, GgCctCCCA
<i>CXCR4</i>	Chemokine (C-X-C motif) receptor 4	↑	+	GACctCCCA, GACCACCCg
<i>DMN</i>	Desmuslin	↓	–	
<i>DNAJB9</i>	DnaJ (Hsp40) homolog, subfamily B, member 9	↓	–	
<i>FOXA1</i>	Forkhead box A1	↓	–	
<i>GATM</i>	Glycine amidinotransferase	↓	–	
<i>KIDINS220</i>	Kinase D-interacting substance 220	↓	–	
<i>MGMT</i>	O-6-methylguanine-DNA methyltransferase	↑	+	GtCCACaCA, GAgCACaCA, GACCActCg, GACctgCCA, GAaCACCCc, GgCgACCCA
<i>MTUS1</i>	Mitochondrial tumor suppressor 1	↓	–	
<i>MYCL1</i>	v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived	↑	+	GACCAgCct, GACCAtgCA
<i>myosin IE</i>	Myosin IE	↓	–	
<i>NPM1</i>	Nucleophosmin 1 (Nucleolar phosphoprotein B23) (Numatrin)	↑	–	
<i>NTRK2</i>	Neurotrophic tyrosine kinase, receptor, type 2	↓	+	cACaACCCA, GgCctCCCA, GAgCActCA, aAtCACCCA, cAgCACCCA
<i>p53</i>	Tumor protein p53		+	
<i>PDE4B</i>	Phosphodiesterase 4B, cAMP specific	↓	–	
<i>PLTP</i>	Phospholipid transfer protein (predicted)	↑	+	GAaCACaCA, aAgCACCCA, GgCctCCCA, GACCActCct, cACCAgCCA, tgCCACCCA, GcCCACtCA
<i>PRKCH</i>	Protein kinase C, eta	↓	+	GACCACCCt, GtCctCCCA, GtCCACaCA, GACCAgCct, GACCACctt, GcCaACCCA, GACCACCCA, GgCCACctA, GgCCACaCA, cAgCACCCA, GgCctCCCA, GACCAgCct, GgCCAgCCA, GtCCACCCA, GtCctCCCA, GgCCACCCc
<i>PRPSAP1</i>	Phosphoribosyl pyrophosphate synthetase-associated protein 1	↑	+	GcCCACaCA, GAgCActCA, ccCCACCCA, GcCctCCCA

Gene abbreviation	Gene name	Expression	Promoter	GLI consensus binding sites
<i>RGS16</i>	Regulator of G-protein signaling 16	↑	–	
<i>SGK</i>	Serum/glucocorticoid regulated kinase	↓	–	
<i>SLC19A1</i>	Solute carrier family 19, member 1	↑	+	GgCCcCCCA, GgCCACCCc, GACCcCCcC, GACCgCCcC, tCCACCCA, GcCCACCCc
<i>SLIT2</i>	Slit homolog 2 (<i>Drosophila</i>)	↓	+	GACCgCCct
<i>TST</i>	Thiosulfate sulfurtransferase	↑	–	

Promoter: (+) indicates that a promoter region has been previously identified in the literature, (–) indicates that a promoter region has not been previously identified. GLI1 consensus binding sites: sites identified in the promoter region are listed. Lower-case indicates a mismatch with the consensus sequence GACCACCCA.

TABLE IIGli consensus binding elements in the regulatory regions of *hBMP-7*, *mKrox-20* and *hNKX6.1*

Gene abbreviation	Gene name	GLI consensus binding sites
<i>BMP-7</i>	Bone morphogenetic protein 7	GACCACC _a A GACC _t CCCA GACCAC _t CA
<i>mKrox-20</i>	Early growth response 2	GACC _g CCCA
<i>NKX6.1</i>	<i>Drosophila</i> homolog of <i>Nkx6.1</i>	GACCACCCA G _g CCACCCA T _t GGTGGTC TGGGTGG _a C

GLI consensus binding sites: sites identified in the promoter region are listed. Lower-case indicates a mismatch with the consensus sequence GACCACCCA.