Noncanonical Regulation of the Hedgehog Mediator GLI1 by c-MYC in Burkitt Lymphoma

Joon Won Yoon, Marisa Gallant, Marilyn LG Lamm, Stephen Iannaccone, Karl-Frederic Vieux, Maria Proytcheva, Elizabeth Hyjek, Philip Iannaccone, and David Walterhouse

Abstract

Although Hedgehog signaling plays a major role in GLI1 transcription, there is now evidence suggesting that other pathways/genes, such as c-MYC, may also regulate GLI1 expression. We initiated studies in Burkitt lymphoma cells, which constitutively express c-MYC due to a chromosomal translocation, to determine whether Hedgehog or c-MYC regulates GLI1 expression. We show that all Burkitt lymphoma cell lines tested express GLI1, PTCH1, and SMO and that five of six Burkitt lymphomas express GLI1. Exposure to Sonic or Indian Hedgehog or cyclopamine (SMO inhibitor) does not modulate GLI1 expression, cell proliferation, or apoptosis in most Burkitt lymphoma cell lines. Sequence analysis of PTCH1, SMO, and SuFu failed to show mutations that might explain the lack of Hedgehog responsiveness, and we did not detect primary cilia, which may contribute to it. We show that c-MYC interacts with the 5’-regulatory region of GLI1, using chromatin immunoprecipitation (ChIP) assay, and E-box-dependent transcriptional activation of GLI1 by c-MYC in NIH3T3 and HeLa cells. The c-MYC small-molecule inhibitor 10058-F4 downregulates GLI1 mRNA and protein and reduces the viability of Burkitt lymphoma cells. Inhibition of GLI1 by GANT61 increases apoptosis and reduces viability of some Burkitt lymphoma cells. Collectively, our data provide evidence that c-MYC directly regulates GLI1 and support an antiapoptotic role for GLI1 in Burkitt lymphoma. Burkitt lymphoma cells do not seem to be Hedgehog responsive. These findings suggest a mechanism for resistance to SMO inhibitors and have implications for using SMO inhibitors to treat human cancers. Mol Cancer Res; 11(6); 604–15. ©2013 AACR.

Introduction

The Hedgehog signaling pathway is activated in a wide variety of human cancers (1, 2). Indeed, it is estimated that up to one-third of all human cancers have active Hedgehog signaling. The GLI1 transcription factor mediates the Hedgehog signal, and expression of GLI1 alone is sufficient to transform cells and induce tumors in mice (3, 4). Inhibition of Hedgehog signaling represents a potentially important therapeutic approach for cancers with active Hedgehog signaling. To date, most inhibitors of the Hedgehog pathway target the smoothened (SMO) protein that functions upstream of GLI1 (5). SMO represents an attractive target for small-molecule agonists and antagonists based on its G-protein–coupled receptor–like structure.

A growing body of evidence suggests that activation of GLI1 in some cancers is not controlled exclusively by Hedgehog signaling but also by other pathways such as RAS and TGF-β (6–8). It is important to identify other upstream regulators of GLI1 that function together with or independent of Hedgehog signaling and may contribute to resistance to Hedgehog pathway inhibitors that target SMO.

Recent reports suggest that c-MYC activates GLI1 in vitro and enhances Hedgehog-induced medulloblastoma formation (9, 10). Previously, we identified putative c-MYC–binding elements (E-boxes) in the 5’-regulatory region of GLI1 (11, 12). On the basis of the fact that c-MYC is constitutively expressed in Burkitt lymphoma as a result of the t(8;14)(q24;q34), we initiated studies to determine (i) whether GLI1 and other Hedgehog pathway components are expressed in Burkitt lymphoma, (ii) whether Hedgehog signaling regulates the level of GLI1 expression in Burkitt lymphoma cells, (iii) whether c-MYC directly regulates the level of GLI1 expression, and (iv) the role of GLI1 in Burkitt lymphoma cells.

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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Materials and Methods

Cell lines and tissue
We obtained Burkitt lymphoma cell lines (BL1596, BL1647, BL1648, BL2392, and BL2625) and MC3T3 mouse preosteoblasts from American Type Culture Collection. Burkitt lymphoma cells were cultured in RPMI-1640 media supplemented with 10% FBS. MC3T3 cells were cultured in ascorbic acid-free α-MEM supplemented with 10% FBS, 100 μM penicillin, and 100 μg/mL streptomycin (Life Technologies). Unstained Burkitt lymphoma slides without other identifiers were obtained for 6 Burkitt lymphoma cases from the Pathology Department at the Ann & Robert H. Lurie Children’s Hospital of Chicago (Chicago, IL). The Office of Research Integrity and Compliance reviewed the studies conducted on human pathologic materials and determined they were exempt from Institutional Board Review and oversight.

Reverse transcription-polymerase chain reaction (RT-PCR)
We isolated total RNA from the cell lines using the Qiagen RNeasy Mini Kit (Qiagen). We carried out RT-PCR using the One-Step RT-PCR Kit (Qiagen) or TaqMan Gene Expression Assay reagents (Applied Biosystems). We conducted 30 to 35 cycles of PCR, including denaturation for 30 seconds, annealing for 30 seconds, and amplification. We conducted 30 to 35 cycles of PCR, including denaturation for 30 seconds, annealing for 30 seconds, and amplification for 1 minute. The following primers were used for PCR: GLI1 sense 5'-AGTGATACGCTCAGGTGAA-3' and GLI1 antisense 5'-GACATTGTAGCAGGTGAC-3', GLI2 sense 5'-AAGGATTGACCCACAGGACG-3' and GLI2 antisense 5'-CCGACTCTACGTCCTGTTT-3', GLI3 sense 5'-CGAACAGATGTCAGCGAGAACGC-3' and GLI3 antisense 5'-AAAAGATTGAAGGAGTTGTA-CTGGG-3', PTCH1 sense 5'-CCCTGAGCACATTCTGAAATCC-3' and PTCH1 antisense 5'-GCGAGAATTGGCAAAACCTTGAG-3', SMO sense 5'-TGGGCTTGTGTCATTACCTTCC-3' and SMO antisense 5'-ATCCTGGTTTGCTCTAGTC-3', IHH sense 5'-CAAGCAGTTCACCACCAATTG-3' and IHH antisense 5'-CTGGTATGACACACGATACGGC-3', Desert Hedgehog (DHH) sense 5'-GCTCTCCTGACCACTACTGCT-3' and DHH antisense 5'-TCGTGGCCCAAATACACCC-3', Sonic Hedgehog (SHH) sense 5'-CAGAGGTGTAAGGAGCAAGTGGTGAAG-3' and SHH antisense 5'-AAAGTGAGGAAGTCCGTGTAGAGCC-3', c-MYC sense 5'-TTCTGTGAGAAGGGCCAGCC-3' and c-MYC antisense 5'-TCACCCAGGCCAAAAGCACGCTTTACTCCATCCGC-3', GAPDH sense 5'-TGATGACATCAAGAAGGTGGTGTGAAG-3' and GAPDH antisense 5'-TCCTGGAGGACCATGGTGCCAT-3'.

Western blot analysis
We prepared Burkitt lymphoma cell lysates using Tris-HCl buffer (pH 7.4), containing 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L dithiothreitol, and 1% TritonX-100. We loaded 50 to 100 μg of protein onto 4% to 15% SDS-PAGE gels (BioRad). After electrophoresis, we blotted the proteins onto nitrocellulose membranes (BioRad) and probed with polyclonal antibodies against human GLI1 protein (Cell Signaling) or against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotech). We visualized the protein using a chemiluminescence kit (Pierce Inc.). For caspase-3 experiments, membranes were probed with caspase-3 or GAPDH antibodies (Cell Signaling) and donkey anti-rabbit IgG-HRP secondary antibody (Santa Cruz Biotech).

MTT assays
We conducted MTT assays as previously described with minor modifications (13). We added 10 μL of MTT reagent (5 mg/mL MTT in 1× PBS) to 0.1 mL of culture media containing the cells. The mixture was incubated for 3 hours at 37°C. 0.1 mL of solubilization solution (10% SDS in 0.01 mol/L HCl) was then added, and the mixture incubated overnight at 37°C. Absorbances at 570 and 650 nm were measured using a Ceres UV900H Di ELISA plate reader (Bio-Tek Instruments, Inc.). Background readings at 650 nm were subtracted from optical density readings at 570 nm. For analysis of growth rate, we added 40,000 cells to a 6-well plate and completed assays on days 0, 2, 4, and 6. The experiments were completed in triplicate and an average and SD calculated.

Immunohistochemistry
We used rabbit anti-GLI1 antibody (Santa Cruz Biotech) for immunohistochemistry. The staining was conducted using a Bond Max Autostainer (Leica-Microsystems) following high pH epitope antigen retrieval (ER2) for 20 minutes. Slides were incubated for 50 minutes with anti-GLI1 antibody. Staining was detected using a refined HRP polymer system (Leica-Microsystems) following by 10-minute diaminobenzidine.

Expression of Hedgehog pathway genes and c-MYC in human B-cell lymphomas
We interrogated publicly available gene expression data from the website http://llmpp.nih.gov/BL/ for statistically significant differences in the expression of c-MYC and Hedgehog pathway genes (GLI1, GLI2, GLI3, SMO, and PTCH1) between Burkitt lymphomas and other diffuse large B-cell lymphomas, including activated B-cell–like lymphomas (ABC), germinal-center B-cell–like lymphomas (GCB), and primary mediastinal B-cell lymphomas (PMBL; ref. 14). Expression data were averaged across available gene probe sets and across lymphoma subtypes. When significant differences in gene expression were found between different lymphomas using the Kruskal–Wallis test, expression values for these genes were further analyzed with Mann–Whitney U post hoc tests. We used Mann–Whitney U tests to make pairwise comparisons between Burkitt lymphoma and ABC, Burkitt lymphoma and GCB, and Burkitt lymphoma and PMBLs. Significance levels were set to 0.0167 as prescribed for Bonferroni correction for the 3 comparisons. The data were rendered as a heatmap in the R statistical programming language (http://www.R-project.org) and arranged according to disease for the GLI1 probeset and 3 c-MYC probesets.
Hedgehog treatment of cells

We exposed Burkitt lymphoma cells and MC3T3 mouse preosteoblasts to SHH peptide (R&D Systems), SHH-containing conditioned media (serum-free conditioned media from confluent cultures of human LNCaP prostate cancer cells, which have been genetically modified to express high levels of SHH, designated LNSh cells; ref. 15), or to IHH peptide (R&D Systems). The cells were incubated for 24 hours at 37°C with serum-free culture media containing 1 μg/mL Hedgehog peptide. For SHH-containing conditioned media treatment, cells were incubated for 3 days at 37°C.

Quantitative RT-PCR
cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems). PCR was carried out using TaqMan universal PCR master mix with reverse transcription kit (Applied Biosystems). PCR was carried out using TaqMan universal PCR master mix with reverse transcription kit (Applied Biosystems). Primers and probes for GLI1 (Hs00171790_ml), PTCH1 (Hs00970980_ml), and GAPDH (Hs09999905_ml) were purchased from Applied Biosystems. The experiments were completed in triplicate and an average and SD calculated.

Cell proliferation assays
We measured cell proliferation using a BrdU cell proliferation assay kit (Chemicon). Cells were incubated with bromodeoxyuridine (BrdUrd) for 1 hour at 37°C following a 24-hour incubation with or without Hedgehog peptide. Cells were fixed and BrdUrd incorporation was measured with a luminometer (Berthold). The experiments were completed in triplicate and an average and SD calculated.

Caspase-3/7 assays
We measured apoptosis using a Promega caspase 3/7 assay kit (Promega). Cells were incubated with an equal volume of 2× caspase assay solution for 1 hour at room temperature in the dark. Caspase activity was measured with a luminometer (Berthold). The experiments were completed in triplicate and an average and SD calculated.

Inhibitor treatment of cells
We added 550,000 cells in 3 mL of RPMI-1640 media supplemented with 10% (v/v) FBS to Falcon 6-well cell culture plates (Becton Dickinson) and added either cyclopamine (SMO inhibitor; LC Lab), tomatidine (negative control for cyclopamine; EMD Bioscience, Inc.), 10058-F4 (c-MYC inhibitor; Sigma), GANT61 (GLI1 inhibitor; Sigma), or equal volumes of solvent [ethanol or dimethyl sulfoxide (DMSO)]. Cells were incubated with the inhibitor at 37°C and 5% CO2 for up to 48 hours. We then carried out quantitative RT-PCR, MTT, BrdUrd, or caspase-3/7 assays as described.

Sequence analysis
We sequenced 23 PTCH1 exons, SMO exons 9 and 10, and SuFu exons 1 and 10. The exons were PCR-amplified from BL1648 gDNA using the following primers: PTCH1-exon 4 sense 5’-GGCGAGCTGCCGGTGTTTATT-3’ and antisense 5’-AGCGTGGGAGAGGCTGTTG-3’, PTCH1-exon 6 sense 5’-CAGGCTGTTGGGGCTCACC-3’ and antisense 5’-GGCTCTAGTGTTGAGGCTGCGC-3’, PTCH1-exon 7 sense 5’-CTATGTGTGATCCAATGGCAGG-3’ and antisense 5’-ATTTAGTAGTGGACCGGCGC-3’, PTCH1-exon 8 sense 5’-AGTGAAGAATTATTGTTCTCCTGCT-3’ and antisense 5’-AGTTTAAGGACACTACTGG-3’, PTCH1-exon 9 sense 5’-GCAAATTTTTCAGGAGACC-3’ and antisense 5’-TGAAACAAACATGATAAGCAAA-3’, PTCH1-exon 10 sense 5’-CCTACAAGTGGATGGC-3’ and antisense 5’-TTTGGCTCTCCACCTTTCTGA-3’, PTCH1-exon 11 sense 5’-GTGACCTGCTGACTAATTTCCC-3’ and antisense 5’-GGCTACGGAGAATAACCGCTTTTA-3’, PTCH1-exon 12 sense 5’-GAGGGCACTGGAAACTTCCC-3’ and antisense 5’-TTTGCAATACACAGGCTGCT-3’, PTCH1-exon 13 sense 5’-TGTCCTGTGAGGCTTGGTG-3’ and antisense 5’-ACGCAGCAGTAAATGGC-3’, PTCH1-exon 14 sense 5’-TGTCCTGTGAGGCTTGGTG-3’ and antisense 5’-ACGGAACAGCAATGATAAGC-3’, PTCH1-exon 15 sense 5’-GTGCTGATGTGCCTGTGGCA-3’ and antisense 5’-CTTAGGAAACAGGAGAAGCTG-3’, PTCH1-exon 16 sense 5’-TCCAGTCTGACCTTCCGC-3’ and antisense 5’-TGCTGGAAGTCAGTGCC-3’, PTCH1-exon 17 sense 5’-GGCGGACGGCATGGTGTGACC-3’ and antisense 5’-GGGAAGACCTTAAGTTGGGCA-3’, PTCH1-exon 18 sense 5’-GCACATTTCCTTCGTTACGC-3’ and antisense 5’-GGGAAGACCTTAAGTTGGGCA-3’, PTCH1-exon 19 sense 5’-GACAAGTTCTCTTTGTGCTCAG-3’ and antisense 5’-ACGAAACAAACATGATAAGCAAA-3’, PTCH1-exon 20 sense 5’-AGGTTCTCTTTGTGCTCAG-3’ and antisense 5’-TCAGTGCTCCACCTTTCTGA-3’, PTCH1-exon 21 sense 5’-AACCAGTTTCCTATGCTTTTTA-3’ and antisense 5’-ACGCCACACCTGACCTTTA-3’, PTCH1-exon 22 sense 5’-GCCAATTTTTCAGGAGACC-3’ and antisense 5’-GCAAATTTTTCAGGAGACC-3’, PTCH1-exon 23 sense 5’-TTGTGACCTTGGACCGGAGGACGCC-3’ and antisense 5’-ACGAAACAAACATGATAAGCAAA-3’.
Noncanonical Regulation of GLI1 by c-MYC

Table 1. Expression of Hedgehog pathway components and c-MYC in Burkitt lymphoma cell lines

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GCCTCACCCTGTCTACGTTTCCCCCTCA-3′ and antisense 5′-GGGGCACGGGCAGTGAAACCAGCTG-3′, SMO-exon 10 sense 5′-TGGCCATGCTGGCCCCCTCAAGA-3′ and antisense 5′-GGGGCACGGGCAGTGAAACCAGCTGTA-3′, SuFu-exon 1 sense 5′-ACAGGGAGGGGGAGGGGGG-3′ and antisense 5′-TCTCCCCGGGGCCCCAGAAGG-3′, SuFu-exon 9 sense 5′-GAGGGCAAGTGGAGACTCTCATC-3′ and antisense 5′-GGGCTCTTGGAGGGCTGGA-3′ (16–20). The products were cleaned using the Wizard PCR Clean-Up Kit (Promega) and sequences were determined using the ABI3730XL DNA analyzer (Applied Biosystems).

Immunofluorescence for cilia

We used Burkitt lymphoma, NIH3T3, and RMS-13 cells in 8-well chamber slides (Nunc). Cells were washed with PBS at room temperature and then fixed with 4% paraformaldehyde/0.5% TritonX-100 in PBS for 30 minutes at room temperature with rocking. Cells were washed again with PBS at room temperature and then blocked for 1 hour with 10% donkey serum. We incubated the cells with anti-acetylated α-tubulin (Sigma; 1:5,000 dilution) and pericentrin2 (Santa Cruz Biotech; 1:500 dilution) antibodies overnight at 4°C. Cells were washed with PBS and incubated with the secondary antibodies at 1:300 dilution (donkey anti-goat IgG Alexa488 and donkey anti-mouse IgG Alexa568) for 1 hour at room temperature. The nuclei were stained with 4′,6-diamidino-2-phenyindole (DAPI) at 1:2,000 dilution (Biotum) for 20 minutes at room temperature with rocking. Immunofluorescence was observed using a Zeiss 510 META confocal laser scanning microscope.

c-MYC–GLI1 interaction by chromatin immunoprecipitation

Chromatin immunoprecipitation was conducted using BL1648 cells and the ChIP-IT Express Chromatin Immunoprecipitation Kit (Active Motif), BL1648 proteins and DNA were cross-linked with formaldehyde for 10 minutes at room temperature. The DNA–protein complexes were sheared by sonication, the cell lysate was cleared by centrifugation at 14,000 × g for 10 minutes, and anti-c-MYC antibody was added (Santa Cruz Biotech). Antibody–protein–DNA complexes were precipitated with protein G magnetic beads. DNA was purified and used for PCR amplification of c-MYC–binding sites. We used the following primers: GLI1, –633 to –447 sense 5′-CCGCTGCTTCTGTGTTTATG-3′ and antisense 5′-TAGGAGTGGTCTGTTTATG-3′, GLI1 364 to 491 sense 5′-ACCGTTGGGTCCTTCTGCCG-3′ and antisense 5′-CACAACCTGGGTTACCTCTC-3′. Cotransfection assays

We cotransfected NIH3T3 or HeLa cells with 0 to 1,000 ng of c-MYC effector plasmid, 500 ng of the Luc-E2 or mutant Luc-E2 GLI1 promoter reporter constructs, and 20 ng of Renilla control reporter DNA (Promega), using 2 to 6 μL of Lipofectamine 2000 reagent (Gibco-BRL). A total of 3 μg of DNA was transfected in each experiment, and the difference was made up with pUC 18 carrier DNA. Cell lysates were prepared 24 hours after transfection. When indicated, cells were concurrently treated with cycloheximide (10 μg/mL; A.G. Scientific, Inc.) during the incubation period. About 20 μL of cell lysate was assayed by adding 100 μL of substrate solution (Promega). Alternatively, quantitative RT-PCR was carried out. The experiments were carried out at least in triplicate and results expressed as an average with SD.

Mutagenesis of E-boxes 3 and 5

The Luc-E2 GLI1 promoter reporter construct, containing mutant E-boxes #3 and #5 was prepared using a site-directed mutagenesis kit (Stratagene) and the following primers: E-box #3, sense 5′-AGGGGGGAGAACCCGCCCTGCGC-3′ and antisense 5′-GACGGCCAGTGGAGACTCTCATC-3′; E-box #5, sense 5′-TAGAGAGGTAACCCAAGGTTTGTGTCTGC-3′ and antisense 5′-CACGGCCAGAACCAGCGGTTTACCTCCTC-3′. DNA was mixed with the primers and 18 cycles of PCR were carried out, including denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and amplification at 68°C for 1 min/kb DNA length. DNA was then digested with DpnI, and the remaining mutated DNA was isolated following bacterial transformation and DNA purification.

Annexin-V staining + FACS apoptosis assays

BL1647, BL1648, and BL2625 cells were seeded onto 48-well plates (0.5 × 10^6 cells/mL) and treated with either vehicle (0.1% ethanol) or increasing amounts of GANT61 for 48 hours. We conducted Annexin-V and 7-aminoactinomycin D (AAD) staining using Guava Nexin Reagent followed by fluorescence-activated cell-sorting (FACS) analysis using Guava EasyCyte (EMD Millipore).

Statistics

Differences between groups were assessed using one-way ANOVA followed by a Student t test unless stated otherwise. P ≤ 0.05 was considered significant.
GLI1 expression in Burkitt lymphoma. A, GLI1 expression by Western blotting in Burkitt lymphoma cells (top). RMS-13 is a GLI1-amplified rhabdomyosarcoma cell line (positive control). GLI1 protein = 150 kDa. The 100 kDa band in the RMS-13 lane is nonspecific. GAPDH was used to show comparable loading of the samples (bottom, GAPDH protein = 37 kDa). More BL1596 protein was loaded than for the other cells. B, growth curves for Burkitt lymphoma cell lines obtained by MTT assay. Solid squares = BL2625 cells, solid circles = BL1648 cells, open circles = BL1596 cells, solid triangles = BL2392 cells, and open squares = BL1647 cells. One-way ANOVA shows differences among the curves (P < 0.05). C, GLI1 immunohistochemistry of Burkitt lymphoma samples. Histologic section of Burkitt lymphoma, showing medium-sized neoplastic cells with multiple small nucleoli and finely dispersed chromatin [hematoxylin and eosin (H&E) stain; a]. Example of an immunohistochemical stain of a Burkitt lymphoma showing strong expression of GLI1 (b). Example of a Burkitt lymphoma that does not express GLI1 by immunohistochemical stain (c). A reactive lymph node does not show expression of GLI1 by immunohistochemical stain (negative control; d). D, gene expression data for GLI1 and c-MYC in 4 types of human lymphoma; Burkitt lymphoma (BL), ABC, GCB, and PMBLs from http://llmpp.nih.gov/BL/ (14). A Kruskal–Wallis test revealed a significant effect of lymphoma subtype on GLI1 [\( \chi^2(3) = 38.3, P < 0.001 \)] and c-MYC [\( \chi^2(3) = 9.0, P = 0.029 \)] gene expression. Mann–Whitney U tests with a Bonferroni correction using a significance level of 0.0167 were used to look for significant differences between disease subtypes in a pairwise manner. For GLI1, comparisons between BL and ABC (U = 53.0, P < 0.001, r = 0.66), BL and GCB (U = 26.0, P < 0.001, r = 0.76), and BL and PMBLs (U = 28.0, P < 0.001, r = 0.75) were all significantly different. The analysis for c-MYC showed a significant difference between BL and GCB (U = 1603.5, P = 0.011, r = 0.22). * indicates significant differences in gene expression between lymphoma subtypes. Note that the ordinate scale is log2. E, gene expression data for GLI1 and c-MYC in the lymphomas listed in Table 1 from http://llmpp.nih.gov/BL/ shown as a heatmap for 1 GLI1 probeset and 3 c-MYC probesets (rows; ref. 14). Red indicates gene upregulation and green downregulation in the heatmap. Each column represents a different patient.

Figure 1. GLI1 expression in Burkitt lymphoma. A, GLI1 expression by Western blotting in Burkitt lymphoma cells (top). RMS-13 is a GLI1-amplified rhabdomyosarcoma cell line (positive control). GLI1 protein = 150 kDa. The 100 kDa band in the RMS-13 lane is nonspecific. GAPDH was used to show comparable loading of the samples (bottom, GAPDH protein = 37 kDa). More BL1596 protein was loaded than for the other cells. B, growth curves for Burkitt lymphoma cell lines obtained by MTT assay. Solid squares = BL2625 cells, solid circles = BL1648 cells, open circles = BL1596 cells, solid triangles = BL2392 cells, and open squares = BL1647 cells. One-way ANOVA shows differences among the curves (P < 0.05). C, GLI1 immunohistochemistry of Burkitt lymphoma samples. Histologic section of Burkitt lymphoma, showing medium-sized neoplastic cells with multiple small nucleoli and finely dispersed chromatin [hematoxylin and eosin (H&E) stain; a]. Example of an immunohistochemical stain of a Burkitt lymphoma showing strong expression of GLI1 (b). Example of a Burkitt lymphoma that does not express GLI1 by immunohistochemical stain (c). A reactive lymph node does not show expression of GLI1 by immunohistochemical stain (negative control; d). D, gene expression data for GLI1 and c-MYC in 4 types of human lymphoma; Burkitt lymphoma (BL), ABC, GCB, and PMBLs from http://llmpp.nih.gov/BL/ (14). A Kruskal–Wallis test revealed a significant effect of lymphoma subtype on GLI1 [\( \chi^2(3) = 38.3, P < 0.001 \)] and c-MYC [\( \chi^2(3) = 9.0, P = 0.029 \)] gene expression. Mann–Whitney U tests with a Bonferroni correction using a significance level of 0.0167 were used to look for significant differences between disease subtypes in a pairwise manner. For GLI1, comparisons between BL and ABC (U = 53.0, P < 0.001, r = 0.66), BL and GCB (U = 26.0, P < 0.001, r = 0.76), and BL and PMBLs (U = 28.0, P < 0.001, r = 0.75) were all significantly different. The analysis for c-MYC showed a significant difference between BL and GCB (U = 1603.5, P = 0.011, r = 0.22). * indicates significant differences in gene expression between lymphoma subtypes. Note that the ordinate scale is log2. E, gene expression data for GLI1 and c-MYC in the lymphomas listed in Table 1 from http://llmpp.nih.gov/BL/ shown as a heatmap for 1 GLI1 probeset and 3 c-MYC probesets (rows; ref. 14). Red indicates gene upregulation and green downregulation in the heatmap. Each column represents a different patient.
Results
Expression of c-MYC and Hedgehog pathway components in Burkitt lymphoma

RT-PCR analysis shows expression of c-MYC, PTCH1, SMO, and GLI1 mRNA in all Burkitt lymphoma cells lines tested (BL1647, BL1648, BL2392, BL2625, and BL1596) and IHH or DHH in a subset of the cell lines (Table 1). The expression patterns suggest that the cells could be Hedgehog-responsive in paracrine or sometimes autocrine manners. Different Burkitt lymphoma cells show variable levels of GLI1 protein and growth rates (Fig. 1A and B). The amount of GLI1 protein detected by Western blotting does not correlate with growth rate. We also show GLI1 expression by immunohistochemistry, showing mostly nuclear staining in 5 of 6 human Burkitt lymphoma samples with MYC translocations (Fig. 1C). Consistent with the variability in GLI1 expression among Burkitt lymphoma cell lines, the degree of GLI1 staining varied by immunohistochemistry with some cases showing strong positivity and others weak staining. We queried publicly available gene expression data for a variety of B-cell lymphomas, including Burkitt lymphoma, activated B-cell–like lymphoma, germinal center B-cell–like lymphoma, and primary mediastinal B-cell lymphoma (14). We found significant upregulation of c-MYC in Burkitt lymphoma compared with germinal center B-cell–like lymphoma and of GLI1 in Burkitt lymphoma compared with all of the other diffuse large B-cell lymphomas (Fig. 1D and E). PTCH1, SMO, GLI2, and GLI3 were not upregulated in Burkitt lymphoma compared with other B-cell lymphomas.

Burkitt lymphoma cell lines are not Hedgehog-responsive
To determine whether Burkitt lymphoma cells respond to ligand-mediated Hedgehog pathway activation, we exposed the cells to either SHH peptide, SHH-containing conditioned media, or IHH peptide. We did not test all of the cell lines in all experiments. For the most part, we did not find changes in GLI1 or PTCH1 expression following exposure of Burkitt lymphoma cells to SHH or IHH (Fig. 2A and B). We detected changes in GLI1 expression only in BL2625 cells exposed to SHH, which are of questionable biologic significance. We show increased GLI1 and PTCH1 expression in MC3T3 mouse preosteoblast, which have been previously shown to be Hedgehog-responsive cells (15). In addition, we do not detect significant changes in cell viability measured by MTT assays, cell proliferation measured by BrdUrd assays, or apoptosis measured by caspase-3/7 assays.

Figure 2. Exposure of Burkitt lymphoma cells to SHH, IHH, or cyclopamine. A, following exposure to SHH peptide or SHH-containing conditioned media, we did not detect changes in the expression of GLI1 (black bars) or PTCH1 (gray bars) mRNA by quantitative RT-PCR in BL1648 or BL1647 cells. We detected a slight increase in GLI1 expression in BL2625 cells ( , P ≤ 0.05). We detect changes in GLI1 and PTCH1 mRNA in the Hedgehog-responsive MC3T3 control cells ( , P < 0.05). C (control) = no SHH (no peptide or SHH-containing conditioned media); P (peptide) = SHH peptide at 1 μg/mL; CM (conditioned media) = SHH-containing conditioned media as described under Materials and Methods. B, following exposure to IHH peptide, we do not detect changes in the expression of PTCH1 (gray bars) or GLI1 (black bars) mRNA by quantitative RT-PCR in BL1648, BL2625, BL1647, or BL1596 cells. We find increased GLI1 and PTCH1 expression in the MC3T3 Hedgehog-responsive control cells ( , P ≤ 0.05). C (control) = no IHH peptide; P (peptide) = IHH peptide at 1 μg/mL. C, MTT assays show statistically significant changes in BL1648 (black bars) and BL2625 cell (white bars) viability but not in BL1596 cell viability (gray bars) following treatment with cyclopamine (left). Changes in viability are also seen in BL1648 cells (black bars), BL1596 cells (gray bars), and BL2625 cells (white bars) following treatment with tomatidine (right; negative control).
following exposure of Burkitt lymphoma cells to SHH or IHH peptide (Supplementary Data).

To determine whether Hedgehog signaling is already highly active and cannot be further stimulated in Burkitt lymphoma cells, we inhibited the pathway with the SMO inhibitor cyclopamine. Inhibition of Hedgehog signaling by cyclopamine does not affect GLI1 mRNA expression in BL1648 or BL1596 cells (Supplementary Data). In addition, cyclopamine treatment does not generally affect Burkitt lymphoma cell proliferation measured by BrdUrd assays or apoptosis measured by caspase-3/7 assays (Supplementary Data). We observe a reduction in BL1648 cell proliferation after treatment with 10 μmol/L cyclopamine, which was not apparent for BL1596 or BL2625 cells and is of uncertain biologic significance. Curiously, cyclopamine reduces Burkitt lymphoma cell viability in BL1648 and BL2625 cell lines (Fig. 2C). Similar results in the same cell lines with the negative control tomatidine combined with the fact that GLI1 expression does not change following treatment with 10058-F4 and cyclopamine does not change c-MYC RNA or protein expression at baseline. c-MYC activates luciferase reporter gene transcription through the 5' regulatory region of GLI1 in NIH3T3 cells and HeLa cells and upregulates GLI1 transcripts in NIH3T3 cells (Fig. 4C–E). Mutations introduced into E-boxes #3 and #5 in the GLI1-regulatory region reduce c-MYC–induced expression. The findings suggest that c-MYC may directly activate GLI1 expression, in part, through E-boxes #3 and #5.

The c-MYC inhibitor 10058-F4 downregulates GLI1 mRNA and protein expression in Burkitt lymphoma cells (Fig. 5A–C). Consistent with our quantitative RT-PCR data (Supplementary Data), cyclopamine does not reduce GLI1 mRNA or protein level. As expected, the GLI1 inhibitor GANT61, which inhibits GLI1-DNA binding, also does not affect GLI1 mRNA or protein expression. In addition, GANT61 and cyclopamine do not affect c-MYC mRNA expression in BL1648 cells. Treatment of BL1648 cells with 10058-F4 also inhibits cell viability (Fig. 5D). Combined treatment with 10058-F4 and cyclopamine does not change BL1648 cell viability compared with treatment with 10058-F4 alone (Fig. 5D). This is consistent with our observation following exposure of Burkitt lymphoma cells to SHH or IHH peptide (Supplementary Data).

As we could not find evidence for up- or downregulation of Hedgehog signaling in Burkitt lymphoma cells despite the expression of Hedgehog pathway components, we looked for mutations in Hedgehog pathway genes (PTCH1, SMO, or SuFu) that might constitutively activate the pathway and prevent us from modulating pathway activity and for primary cilia that may play a role in mediating Hedgehog signaling. We sequenced 23 PTCH1 exons in BL1648 cells and did not find any mutations. We also sequenced known hotspots for activating SMO mutations (exons 9 and 10) or inactivating SuFu mutations (exons 1 and 9) and did not find any mutations (21, 22). Therefore, we do not find evidence for mutations that might constitutively activate the pathway and could account for the lack of Hedgehog responsiveness. In some cells, Hedgehog signaling may be mediated through primary cilia (23–27). A primary cilium is a microtubule-containing organelle that projects from the cell. It attaches to the cell at a microtubule-organizing center, which includes a pair of centrioles (28). It is generally accepted that hematopoietic cells do not have primary cilia. It is less clear whether lymphoma cells have primary cilia; therefore, we looked for primary cilia in Burkitt lymphoma cells. We did not detect primary cilia projecting from BL1648 cells (Fig. 3) or BL2625 cells (data not shown) by immunofluorescence, which may contribute to the lack of Hedgehog responsiveness in these cells. However, we detected colocalization of acetylated α-tubulin and pericentrin2 in BL1648 and BL2625 cells in a nonprojectile pattern more consistent with an immune synapse that has been described in lymphoid cells (29).

### c-MYC regulates the expression of GLI1

Previously, we recognized that the 1,492-bp 5′-regulatory region of GLI1 contains 5 E-boxes (CANNTG) that represent potential c-MYC–binding sites (Fig. 4A; refs. 11, 12). c-MYC physically interacts with the 5′-regulatory region of GLI1 in vivo (Fig. 4B).

As Burkitt lymphoma cells already have high c-MYC expression and as hematopoietic cells are difficult to transfect, we tested the effect of c-MYC upregulation on GLI1 expression in cells with low endogenous c-MYC expression at baseline. c-MYC activates luciferase reporter gene transcription through the 5′-regulatory region of GLI1 in NIH3T3 cells and HeLa cells and upregulates GLI1 transcripts in NIH3T3 cells (Fig. 4C–E). Mutations introduced into E-boxes #3 and #5 in the GLI1-regulatory region reduce c-MYC–induced expression. The findings suggest that c-MYC may directly activate GLI1 expression, in part, through E-boxes #3 and #5.

Figure 3. Detection of primary cilia in cell lines. Immunofluorescence detects primary cilia projecting from NIH3T3 (acetylated α-tubulin antibody (red) detects the primary cilium, DAPI (blue) shows the nucleus; left, top) and RMS-13 cells (acetylated α-tubulin antibody (red) detects the primary cilium, pericentrin2 antibody (green) detects the microtubule organizing center, and DAPI (blue) shows the nucleus; left, bottom) but not in BL1648 cells (acetylated α-tubulin antibody (red) and pericentrin2 (green) colocalize consistent with an immune synapse but do not show a projecting primary cilium, DAPI (blue) shows the nucleus; right). BL1648 cells maintained in serum and serum-starved cells are shown. All scale bars = 10 μm.
that SMO inhibition does not regulate GLI1 expression in Burkitt lymphoma cells. Taken together, these results suggest that c-MYC directly regulates GLI1 expression in a variety of cell types including Burkitt lymphoma cells.

**GLI1 inhibits apoptosis in Burkitt lymphoma cells**

To understand the role of GLI1 in Burkitt lymphoma cells, we treated the cells with the GLI1-inhibitor GANT61 and assessed cell viability, cell proliferation, and apoptosis. Treatment with GANT61 consistently increases apoptosis (Fig. 6C–E) and reduces cell viability in BL1648 cells and BL1596 cells (Fig. 6A). However, treatment with GANT61 does not generally alter the proliferation of Burkitt lymphoma cells (Fig. 6B). Therefore, GLI1 functions as an anti-apoptotic factor in Burkitt lymphoma cells and enhances viability of some Burkitt lymphoma cells. Of interest, BL1648 and BL1596 cells express higher GLI1 levels on Western blotting (Fig. 1A) compared with BL2625 cells that do not show reduced viability when treated with GANT61. Changes in Bcl2 expression with GANT61 treatment of Burkitt lymphoma cells were not detected, and at this point, the antiapoptotic mechanism is uncertain.

**Summary**

Burkitt lymphoma cells express both c-MYC and Hedgehog pathway components, including *PTCH1*, *SMO*, *GLI1*,
and sometimes IHH or DHH. However, Burkitt lymphoma cells are not Hedgehog responsive. SHH or IHH do not increase PTCH1 and GLI1 mRNA expression in most Burkitt lymphoma cell lines and do not affect cell viability, cell proliferation, or apoptosis. The Hedgehog pathway inhibitor cyclopamine does not generally affect GLI1 expression, cell proliferation, or apoptosis. In contrast, c-MYC directly binds the GLI1 promoter region and regulates GLI1 expression in a variety of cell types including Burkitt lymphoma. Finally, GLI1 functions as an antiapoptotic factor in Burkitt lymphoma cells. Taken together, c-MYC regulates GLI1 expression in Burkitt lymphoma more significantly than the canonical Hedgehog signaling pathway. This finding has therapeutic relevance and suggests that GLI1 or...
GLI1 target genes may be better therapeutic targets than SMO in Burkitt lymphoma and potentially other cancers that express GLI1 and c-MYC.

**Discussion**

We show that Burkitt lymphoma cells express both c-MYC and Hedgehog pathway components. For the most part, activation or inhibition of the Hedgehog pathway does not seem to alter expression of GLI1, the mediator of Hedgehog signaling, or proliferation or apoptosis of Burkitt lymphoma cells. Conversely, we show that c-MYC binds the GLI1 regulatory region and regulates GLI1 expression. Our results suggest that GLI1 expression in Burkitt lymphoma cells may be largely driven by c-MYC instead of canonical...
Hedgehog signaling. This is supported by publicly available gene expression data for a variety of B-cell lymphomas that show significant upregulation of c-MYC and GLI1 but not other Hedgehog pathway components in Burkitt lymphoma compared with other B-cell lymphomas (14). Ultimately, GLI1 seems to function as an antiapoptotic factor in Burkitt lymphoma cells. Identifying mechanisms for GLI1 activation other than the canonical Hedgehog signaling pathway is relevant to developing therapeutic approaches that target Hedgehog signaling in a variety of cancers. Most agents currently under investigation target SMO, upstream of GLI1, and therefore might not be effective in settings where GLI1 expression is driven by other mechanisms.

Active paracrine Hedgehog signaling has been shown in other B-cell malignancies (30, 31). Hedgehog pathway components are present in Burkitt lymphoma cells. The reason we could not show modulation of Hedgehog signaling by either exposing cells to Hedgehog ligands or cyclopamine is not clear. We did not identify mutations in Hedgehog pathway components that could prevent us from altering activity by constitutively regulating pathway output. In addition, although we could identify primary cilia in NIH3T3 cells and RMS-13 cells following serum starvation, we did not identify primary cilia in the Burkitt lymphoma cells even following serum starvation. Absence of primary cilia could contribute to the lack of Hedgehog-responsive signaling in these cells. Controversy continues regarding the role of primary cilia in Hedgehog signaling in cancer cells (23–27). It is possible that primary cilia may be present in Burkitt lymphoma in vivo.

It is intriguing that some Burkitt lymphoma cells show reduced viability when exposed to cyclopamine. The fact that GLI1 expression is not altered by exposure to cyclopamine; the same cells show reduced viability when exposed to the negative control compound tomatidine; and that apoptosis, which seems to be regulated in part by GLI1 in Burkitt lymphoma, was not affected by exposure to cyclopamine suggest that other mechanisms may account for the reduced viability. It remains unclear why some Burkitt lymphoma cell lines show reduced viability while others do not.

We show that altering c-MYC expression or activity consistently affects GLI1 expression in several types of cells, including Burkitt lymphoma. In addition, inhibition of c-MYC activity with 10058-F4 reduces the viability of Burkitt lymphoma cells. In BL1648 cells, this is not affected by simultaneous treatment with cyclopamine. As inhibition of GLI1 function with GANT61 reduces the viability of BL1648 cells, we might expect that inhibition of active Hedgehog signaling with cyclopamine together with inhibition of c-MYC could have additive or even synergistic effects on cell viability. Taken together, our findings suggest that c-MYC impacts the expression of GLI1 in Burkitt lymphoma to a larger extent than canonical Hedgehog signal transduction.

We show that GLI1 functions as an antiapoptotic factor in Burkitt lymphoma. It is therefore not surprising that reducing GLI1 activity is sometimes also associated with reduced cell viability in cells that have high GLI1 expression. Our results suggest that Hedgehog pathway inhibitors that are directed at SMO and currently in clinical trials for other cancers may not be effective in settings where there is noncanonical activation of GLI1. GLI1 or GLI1 target genes represent potential therapeutic targets in Burkitt lymphoma.

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Noncanonical Regulation of the Hedgehog Mediator GLI1 by c-MYC in Burkitt Lymphoma

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