Gli1 Protein Regulates the S-phase Checkpoint in Tumor Cells via Bid Protein, and Its Inhibition Sensitizes to DNA Topoisomerase 1 Inhibitors*

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Background: Aberrant expression of Gli1 is observed in cancers of many tissues and is associated with aggressive disease.

Results: Gli1 inhibition in tumor cells abrogates ATR-mediated Chk1 phosphorylation by down-regulating the BH3-only protein Bid and sensitizes them to camptothecin.

Conclusion: Gli1 inhibition sensitizes tumor cells to chemotherapy.

Significance: These results identify a novel mechanism of Gli1-mediated S-phase checkpoint regulation and therapeutic combination.

Aberrant expression of hedgehog molecules, particularly Gli1, is common in cancers of many tissues and is responsible for their aggressive behavior and chemoresistance. Here we demonstrate a novel and tumor-specific role for aberrant Gli1 in the regulation of the S-phase checkpoint that suppresses replication stress and resistance to chemotherapy. Inhibition of Gli1 in tumor cells induced replication stress-mediated DNA damage response, attenuated their clonogenic potential, abrogated camptothecin (CPT)-induced Chk1 phosphorylation, and potentiated its cytotoxicity. However, in normal fibroblasts, Gli1 siRNAs showed no significant changes in CPT-induced Chk1 phosphorylation. Further analysis of ataxia telangiectasia and Rad3-related protein (ATR)/Chk1 signaling cascade genes in tumor cells revealed an unexpected mechanism whereby Gli1 regulates ATR-mediated Chk1 phosphorylation by transcriptional regulation of the BH3-only protein Bid. Consistent with its role in DNA damage response, Bid down-regulation in tumor cells abolished CPT-induced Chk1 phosphorylation and sensitized them to CPT. Correspondingly, Gli1 inhibition affected the expression of Bid and the association of replication protein A (RPA) with the ATR-interacting protein (ATRIP)-ATR complex, and this compromised the S-phase checkpoint. Conversely, complementation of Bid in Gli1-deficient cells restored CPT-induced Chk1 phosphorylation. An in silico analysis of the Bid promoter identified a putative Gli1 binding site, and further studies using luciferase reporter assays confirmed Gli1-dependent promoter activity. Collectively, our studies established a novel connection between aberrant Gli1 and Bid in the survival of tumor cells and their response to chemotherapy, at least in part, by regulating the S-phase checkpoint. Importantly, our data suggest a novel drug combination of Gli1 and Top1 inhibitors as an effective therapeutic strategy in treating tumors that express Gli1.

Hedgehog (Hh) signaling through Gli transcription factors plays an essential role during development by regulating many different signaling cascades that control cell division, growth, differentiation, and cell death (1–3). However, Gli expression is minimal or undetectable in differentiated tissues. On the other hand, aberrant expression or mutations of Hh signaling genes are responsible for several human diseases, syndromes, and malformations (4–7). Importantly, aberrant expression of Glis is common in cancers of many tissues, including that of the colon, lungs, ovaries, and pancreas (6). Depending on the stage of development, these cancers often acquire an aggressive and metastatic phenotype and exhibit resistance to chemotherapeutic agents (8, 9). The canonical Hh pathway is a multicomponent signaling cascade (Hh, Patch, Smo) that leads to the expression of downstream effectors Gli1 and Gli2. Activation of these transcription factors regulates the expression of their respective targeted genes (2, 10). However, ligand-independent and non-canonical expression of Gli1 is also known in many cancers and may be important in augmenting specific oncogenic and growth signals (11–14). Therefore, agents that target Smoothened (Smo) or upstream targets of the Hh pathway remain largely ineffective in these cancers (15, 16), suggesting the importance of the development of small molecule inhibitors that may target Glis, particularly Gli1. Although Gli1 is often an up-regulated Hh component in many tumors, identification of the signaling mechanisms it regulates in tumor cells is critical in developing effective therapeutic modalities (17–20). Recently, several studies indicated the influence of Hh/Gli1...
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signaling on DNA damage response (DDR)\(^3\) and repair pathways to promote more aggressive disease and tumor resistance to therapeutics (21–24). Gli1 expression through canonical Hh signaling or its ectopic expression is linked to genomic instability and cancer predisposition because of the defects in the S-phase checkpoint and DNA repair mechanisms (25, 26). Together, these studies indicate that Gli1 expression influences cellular signaling and its fate. Interestingly, the pharmacological inhibition of non-canonical Gli in colon cancer has been shown to induce DNA double strand breaks (DSBs) and cell cycle arrest (27). However, the mechanism by which Gli1 controls cell cycle progression and by which its inhibition induces DSB-mediated DDR is not completely understood.

DNA topoisomerase 1 (Top1)-inhibiting anticancer agents such as camptothecin (CPT) analogues (irinotecan and topotecan) show a broad spectrum of antitumor activity against colorectal, lung, and ovarian cancer either alone or in combination with other agents (28–30). The therapeutic activities of CPT and its analogues are attributed to prolonged stabilization of Top1-DNA covalent complexes (Top1cc) and conversion of them into lethal DNA lesions during S-phase (30, 31). The prolonged stabilization of Top1cc by CPT not only physically hinders fork progression but also inhibits the relaxation of positive supercoils generated ahead of the replication fork, and such events generate replication stress and replication-coupled DSBs (31, 32). Although ataxia telangiectasia mutated (ATM)/Chk2 and ATR/Chk1 kinase-mediated signaling cascades are activated in response to CPT treatment, ATR/Chk1-mediated responses play a predominant role in orchestrating DDR signaling and cell survival (30, 33–36). These effects can be attributed to the generation of replication stress and replication-coupled DSBs because of Top1 poisoning by CPT (37). The primary response to the replication block is mediated by the activation of ATR-mediated signaling in association with several factors (RPA, ATRIP, clasin1, etc.), leading to phosphorylation of Chk1 (38–42). The replication checkpoint mediated by Chk1 kinase signaling inhibits DNA replication at the level of initiation to prevent excess DNA damage and to activate the G2 checkpoint to provide ample time to repair the DSBs generated from collapsed forks (33, 43–45). Accordingly, the inhibition of ATR/Chk1 signaling enhances the CPT-induced DDR and hypersensitizes cancer cells to Top1 inhibitors (33, 36).

In this study, we elucidated the mechanisms that generate DSB-mediated DDR in cancer cells upon Gli1 inhibition. Our data revealed that transient down-regulation of Gli1 affects ATR-mediated Chk1 phosphorylation in response to CPT in tumor cells but not in primary and untransformed fibroblasts. Because CPT is known to elicit ATR-mediated Chk1 phosphorylation in cells within minutes of exposure, our studies were focused on CPT-induced DDR. We also show that Gli1 inhibition modifies the interaction between RPA and the ATRIP-ATR complex by transcriptional regulation of the BH3 domain protein Bid. These results reveal a novel role for Bid in CPT-induced Chk1 phosphorylation and its regulation by Gli1 in tumor cell survival. Together, these studies identify a novel role for Gli1 in the regulation of the S-phase checkpoint in tumor cells, implying that its aberrant expression may promote their survival during oncogenic replication stress, a common feature of tumor cells, and resistance to drugs that inhibit DNA replication. Importantly, our studies also demonstrate that the inhibition of Gli1 in combination with Top1-targeting agents would be an effective therapeutic modality in Gli1-expressing tumors.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—A549 cells (a human lung adenocarcinoma epithelial cell line), H1299 cells (a human nonsmall cell lung carcinoma cell line), HT29 cells (a colorectal adenocarcinoma cell line), and BJ cells (infant foreskin fibroblasts) were obtained from the ATCC. HDF cells (neonatal human dermal fibroblasts transformed with hTERT) have been described previously (46), and the isogenic ovarian carcinoma cells A2780 (cisplatin-sensitive) and A2780/CP70 (cisplatin-resistant) were a gift from Dr. Eddie Reed (27). A549, H1299, and HDF cells were cultured in Dulbecco’s modified Eagle’s medium, HT29 cells were cultured in McCoy’s medium, BJ cells were cultured in Eagle’s minimal essential medium, and A2780 and A2780/CP70 cells were cultured in RPMI medium as described previously (47). All media were supplemented with 10% FBS, 100 μg/ml streptomycin sulfate, and 100 units/ml penicillin. Cells were routinely tested for mycoplasma contamination using the Mycotest kit (Invitrogen), and cells within 15 passages were used in the experiments. Camptothecin and GANT61 (Sigma) were dissolved in anhydrous DMSO at 10 and 100 mM concentration, respectively, and stored at −20 °C. These stock solutions were further diluted into anhydrous DMSO before addition to the cells. The Bid expression vector pSFV–neo Bid human (Addgene, plasmid no. 8766) has been described previously (48). Antibodies to the following antigens used in this study included the following: ATR, Chk1, BH3 interacting-domain death agonist (Bid) ATRIP, Cdc25A, Claspin, Rad9, Gli1, and GAPDH (Santa Cruz Biotechnology, Inc.); RPA70 and phospho-RPA32 (S4/8) (Bethyl Laboratories, Inc.); phospho-ATM (Ser-1981), phospho-Chk1 (Ser-17 and Ser-345) (Cell Signaling Technology); and γH2AX (Millipore).

**RNA Interference**—The siRNAs used in this study were purchased from Dharmacon and transfected using Lipofectamine 2000 (Invitrogen) according to the protocols of the manufacturer and as described previously (37). The siRNAs used in this study were as follows: siControl (37), siGli1–1, siGli1–2, siGli1–5 (49), and siBid (50). For Gli1 down-regulation, the data using siGli1–2 sequences were presented in these studies unless mentioned otherwise.

**Clonogenic Survival Assays**—Cells were transfected with the indicated siRNAs, plated in 6-well dishes in triplicates, allowed to attach for 16 h, and treated with the indicated concentrations of CPT for another 16–24 h. Following CPT treatment, cells were washed three times with PBS and two times with growth medium (CPT-free) and allowed to form colonies in complete growth medium. After 10–14 days, colonies were fixed in
methanol and stained with crystal violet, and then the colonies with $\geq 25$ cells were counted using an imaging system (Gene Tools, Syngene).

**Immunofluorescence and Western Blotting**—Cells transfected with siRNAs were trypsinized after 48 h and seeded into 35-mm glass bottom dishes. The resulting cells were treated with CPT (or DMSO for controls) at the concentrations specified in the figures or figure legends. Permeabilized cells were fixed in 3% formaldehyde for 10 min and then in 100% methanol ($-20 \, ^{\circ}\text{C}$) for 10 min at room temperature. Fixed cells were blocked in 10% FBS for 30 min. After three washes with PBS, cells were incubated overnight at 4 \, ^{\circ}\text{C} with primary antibodies in PBS containing 5% BSA and 0.1% Triton X-100 (PBS-T). The slides were washed three times with PBS-T containing 1% BSA, incubated with anti-mouse IgG-Cy3 or anti-mouse IgG-FITC antibodies (Molecular Probes) for 2 h at room temperature, and mounted with Vectashield containing DAPI. For Western blotting, cell lysates were prepared after washing extensively with PBS. Cells were lysed in ice-cold cytoskeletal buffer (10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl$_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM ATP, 1 mM Na$_3$VO$_4$, 10 mM NaF, and 0.1% Triton X-100) freshly supplemented with protease and phosphatase inhibitors (Roche). After normalizing the protein concentrations, samples were prepared in 4× SDS-PAGE sample buffer and heated to 100 \, ^{\circ}\text{C} for 15 min. Denatured samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes.

**RNA Isolation and RT-PCR**—Total RNA was extracted from cells at the indicated time intervals, and 1 \, $\mu$g of RNA was reverse-transcribed using a Superscript II reverse transcriptase kit (Invitrogen) according to the protocol of the manufacturer. The amplification of PCR products was quantitated using SYBR Green dye (ABI), and fluorescence was monitored on a CFX96 Bio-Rad sequence detection system. A melting curve analysis was done for each amplicon. The $2^{-\Delta\Delta\text{Ct}}$ method was used for quantitation with GAPDH as an endogenous control. The analysis for each gene was done in triplicate, and three independent biological replicates were performed. The gene-specific primers used for the analysis were purchased from Bio-Rad.

**Flow Cytometry and Intra-S-phase Checkpoint Analysis**—After 48 h of siRNA transfection, A549 cells were trypsinized, fixed in 70% methanol, and analyzed by flow cytometry after propidium iodide staining. To assess the effect of Gli1 on replication, cells were pulse-labeled with 10 \, $\mu$M BrdU for 30 min and fixed as mentioned above. Additionally, cells were incubated with FITC-conjugated anti-BrdU antibody and analyzed by flow cytometry. For the intra-S-phase checkpoint, cells were plated in 24-well culture dishes. The resulting cultures were treated with the indicated concentrations of CPT in the presence of 1 \, $\mu$M $[^{3}\text{H}]$thymidine for 1 h, and the rate of DNA synthesis was determined by measuring $[^{3}\text{H}]$thymidine incorporation (51) using a liquid scintillation counter (Hydex). For each siRNA, rates of DNA synthesis were normalized and expressed as a percent of $[^{3}\text{H}]$thymidine incorporation. Each data point represents a mean of triplicates.

**Immunoprecipitation Assays**—A549 cells were transfected with siRNAs (control and Gli1) twice with a 24-h interval. 48 h post-transfection, cells were treated with DMSO or 500 nM CPT for an additional 2 h. The cells were washed thrice using cold PBS and incubated with cold radioimmune precipitation assay buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSE, 20 mM NaF, 1 mM sodium vanadate, and protease inhibitors for 30 min on ice. The cell lysates were centrifuged for 30 min at 30,000 \times \text{g}. The precleared samples were immunoprecipitated with primary antibody (anti RPA70) overnight followed by incubation with protein A-Sepharose beads, and the interacting proteins were analyzed by Western blotting.

**Sister Chromatid Exchange (SCE) Assays**—48 h post-transfection with control or Gli1 siRNAs, cells were labeled with 10 \, $\mu$M BrdU (Sigma) for two cell cycles, and colcemid was added for last 2 h. Cells were harvested and treated in hypotonic solution (0.075 \, M KCl) for 20 min and subsequently fixed in Carnoy’s fixative (methanol-acetic acid, 3:1) and deposited on slides. SCEs were counted after fluorescence-plus-Giemsa staining as described previously (52). At least 50 metaphases were analyzed, and their mean SCEs per metaphase ± S.E. were calculated for each sample.

**Luciferase Assay**—The luciferase control (catalog no. S790005) and bid promoter (catalog no. S719427) were purchased from Switchgear Genomics. The luciferase assay was performed using the LightSwitch™ luciferase assay system kit according to the protocol of the manufacturer (Promega). Measurements were taken in a Hitachi Plate Chameleon V instrument with a 100-ms delay and 2 s of counting time.

**Statistical Analysis**—The clonogenic survival data presented are averages of three independent experiments performed in triplicates each time. Error bars represent the mean ± S.D. Data were analyzed either by GraphPad Prism 6 or Excel 2010. The data presented in the manuscript are representative of at least two independent experiments.

**RESULTS**

**Transient Down-regulation of Gli1 in Cancer Cells Inhibits Cell Growth and Induces Replication Stress-mediated DDR**—Aberrant expression of Gli1 has been linked to aggressive tumor growth, increased tumor cell survival, and resistance to chemotherapy (21–24). Inhibition of Gli1 has also been shown to induce DDR and to attenuate cell survival. However, the mechanism for Gli1 inhibition-mediated DDR is not known (53, 54). To investigate the role of Gli1-mediated signaling in tumor cell survival and DDRs, we transiently down-regulated Gli1 in several cancer cells using siRNAs and evaluated for the formation of γH2AX foci (a surrogate marker for DSB and replication stress) and their clonogenic potential. As shown in Fig. 1, Gli1 siRNA-transfected A549 cells exhibited a severalfold increase in γH2AX foci compared with control or non-targeting siRNA-transfected cells (Fig. 1, A and B) and attenuated their clonogenic potential (Fig. 1C). Similar results were also observed with H1299 and HT29 cells (data not shown). Consistent with this DDR, Gli1-depleted cells also exhibited an increased rate of spontaneous SCE compared with control siRNA-transfected cells (Fig. 1D). Together, these data indicate that the presence of replication stress or S-phase-specific DNA lesions in Gli1 down-regulated cells could be responsible for
increased spontaneous SCE as a result of homologous recombination-mediated repair of these lesions (55).

Gli1 inhibition abrogates Chk1 phosphorylation and sensitizes cancer cells to CPT—A common feature in cancer cells is proliferation and oncogene signal-mediated replication stress, which is known to induce DDR. During replication stress, the S-phase checkpoint plays a critical role in stabilizing stalled replication forks and in facilitating the repair of DSB generated because of the fork collapse. Therefore, a defect in ATR/Chk1-mediated signaling induces spontaneous DSBs because of endogenous fork-stalling lesions (39). To explore whether Gli1-mediated signaling has any role in the suppression of replication stress-mediated DDR, we transfected A549 and HT29 cells with control or Gli1 siRNAs and treated them with the replication-mediated DSB-inducing agent CPT as a positive control. Consistent with the focus data from immunofluorescence studies (Fig. 1A), increased γH2AX and phosphorylated ATM were observed in Gli1-depleted A549 cells (Fig. 2A). CPT treatment activated the ATR-mediated replication checkpoint response, as expected, leading to phosphorylated Chk1 and induced replication-coupled DSBs, as evidenced by the phosphorylation of ATM and γH2AX in control siRNA-transfected A549 cells (Fig. 2A) and HT29 cells (Fig. 2B). Surprisingly, a substantial reduction in CPT-induced Chk1 phosphorylation (Ser-317 and Ser-345) was observed in Gli1 down-regulated tumor cells of different tissue origins (Figs. 2, A and B, and 5, A and B). Because this was unexpected, we further evaluated whether these defects in CPT-induced Chk1 phosphorylation are due to the differences in ATR and Chk1 protein levels in Gli1-depleted tumor cells. However, no significant differences of Chk1 and its upstream kinase ATR proteins were noted in Gli1-deficient cells (Fig. 2, A and B, and data not shown). Indeed, ATR protein levels were increased significantly in Gli1-depleted cells, ruling out the involvement of Gli1-mediated down-regulation of these proteins (Fig. 2, A and B). To eliminate the possibility of Gli1 down-regulation-mediated effects on the ability of the cells to synthesize DNA, control and Gli1 siRNAs-transfected cells were compared for the number of cells actively synthesizing DNA by BrdU incorporation. The FACS analysis of BrdU versus propidium iodide staining showed only about a 5%, but insignificant reduction in BrdU-positive cells following Gli1 depletion compared with control cells (Fig. 3A). These data suggest that Gli1 depletion does not mediate effects on Chk1 phosphorylation as a result of perturbation in the S-phase cell population.
Moreover, in Gli1-deficient cells, an increased population of early and mid-S-phase BrdU-positive cells were observed compared with control cells (Fig. 3, bottom panel, FITC versus Count). These distinct distributions in replicating cells, the presence of DDR, and increased spontaneous SCEs indicate the persistence of replication stress in Gli1 knockdown cells. The intra-S-checkpoint mediated by Chk1 kinase protects cells from replication stress-mediated DDR, and a defect in Chk1 phosphorylation in these cells could be attributed to increased DNA damage and reduced cell survival.

It is evident from several studies that Chk1 plays a key role in the regulation of the stronger S-phase checkpoint in response to Top1-inhibiting anticancer agents such as CPT and tumor resistance to its clinical analogues (33, 36). Therefore, inhibition of Chk1 kinase by pharmacological inhibitors or its signal depletion sensitizes cancer cells to CPT. To examine whether Gli1 inhibition sensitizes tumor cells to CPT, we further evaluated survival by colony formation. Consistent with the defect in Chk1 phosphorylation, Gli1 knockdown cells exhibited a 2- and 3-fold decrease in the survival of A549 and HT29 cells, respectively, at 1 nM CPT. Similarly, the IC_{50} values for CPT were reduced from 5 to 1 nM for A549 cells and from 10 to 2.5 nM for HT29 cells because of the Gli1 depletion (Fig. 2, C and D). Similar responses were also observed in A2780 and A2780/CP70 ovarian cancer cells (data not shown). To further rule out that Gli1 inhibition-mediated S-phase checkpoint defects are not due to the off-target effects of siRNAs, we used two additional siRNAs that target Gli1 at different sequences. All three Gli1 siRNAs exhibited similar defects in CPT-induced Chk1 phosphorylation, excluding the possibility of off-target effects (Fig. 3B).

Gli1 Inhibition-mediated Defects in Chk1 Phosphorylation Are Specific to Cancer Cells—To test whether Gli1 inhibition-mediated abrogation of Chk1 phosphorylation is specific to cancer cells or it also occurs in normal cells, non-immortalized BJ cells and human telomerase immortalized HDF cells were transfected with control and Gli1 siRNAs and assessed for CPT-induced Chk1 phosphorylation. We did not observe any
detectable levels of Gli1 protein (Fig. 2, E and F) or mRNA in these cells (data not shown). Consistent with the absence of Gli1 expression, no defects in CPT-induced Chk1 phosphorylation were observed in both BJ and HDF cells when control siRNAs and Gli1 siRNAs transfected cells were compared. These data confirm that the aberrant expression of Hh/Gli1 signaling in tumor cells promotes their survival and chemoresistance, at least in part, by regulating the S-phase checkpoint.

Gli1 Knockdown Abrogates the S-phase Checkpoint Mediated by ATR-Chk1 and Induces Cancer Cell Death—To examine whether depletion of Gli1 in tumor cells also inhibits the Chk1-mediated S-phase checkpoint, a signaling response that transiently inhibits DNA synthesis in response to DNA damage was assessed by measuring [3H]thymidine incorporation into DNA. As shown in figure 4A, increased levels of CPT-resistant DNA synthesis were observed in Gli1 knockdown A549 cells compared with control siRNA-transfected cells. This CPT-resistant DNA synthesis in Gli1-inhibited cells resembles the trends observed with caffeine-treated cells, an inhibitor of ATM/ATR kinases that exhibits CPT- and radiation-resistant DNA synthesis.

To further evaluate whether Gli1 inhibition-mediated DDR and cell death are due to the S-phase checkpoint-mediated DDR, we assessed the damage-induced formation of RPA foci. Consistent with the spontaneous γH2AX foci (Fig. 1A), Gli1 down-regulated cells exhibited RPA foci (Fig. 4B). Additionally, exposure to CPT increased the number of foci per cell compared with DMSO-treated cells (Fig. 4B). To further confirm that these DDRs are replication-mediated, control and Gli1-depleted A549 cells were labeled with EdU, exposed them to CPT, and assessed for the number of cells undergoing cell death, as evidenced by sub-G0/G1 cells (Fig. 4D, red line). Consistent with this and the CPT-resistant DNA synthesis data (Fig. 4A), the number of γH2AX foci colocalized with EdU in response to CPT increased significantly compared with DMSO treatment in Gli1 down-regulated cells (Fig. 4C). These results
suggest that the S-phase checkpoint defect in Gli1 knockdown cells resulted in the potentiation of CPT-induced DDR, resulting in a significant increase in RPA and γH2AX foci compared with control cells treated with CPT (Fig. 4, B and C). Together, these data demonstrate that Gli1 inhibition in cancer cells leads to S-phase checkpoint abrogation and sensitizes tumor cells to Top1 inhibitors by augmenting the formation of replication-mediated DSBs.

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FIGURE 4. Gli1 down-regulation inhibits the S-phase checkpoint, induces CPT-resistant DNA synthesis, and increases the number of replication-coupled DSBs. A, control and Gli1 down-regulated A549 cells were treated with the indicated concentrations of CPT or DMSO, and the rate of DNA synthesis was assessed by [3H]thymidine incorporation. Data mean ± S.D. of triplicates (the error bars are too small to be seen). B, control and Gli1 down-regulated A549 cells were exposed to 500 nM CPT and assessed for phospho-RPA (S4/8) foci or added to EdU for 30 min and exposed to CPT to assess the replication-coupled DSBs. C, γH2AX foci (green) colocalized with EdU (red) as a replication marker. D, A549 cells transfected with control and Gli1 siRNAs were exposed to 500 nM CPT for 2 h and allowed to recover for another 24 h in drug-free medium and assessed by flow cytometry. An increased number of sub-G0 cells was observed in CPT-exposed Gli1 knockdown cells after 24 h (red line).

To identify the putative genes that are altered in Gli1 down-regulated cells responsible for the Chk1 phosphorylation defect, we first searched for various genes associated with this signaling mechanism from the literature and assessed their transcript levels using a custom-designed RT-PCR array using their respective primers (Bio-Rad) (data not shown). The gene expression data revealed no significant effect of Gli1 status in the expression of most genes implicated in the ATR/Chk1 cascade. Surprisingly, a substantial decrease in transcript levels of the proapoptotic gene BID (BH3-interacting domain death agonist) was observed in Gli1 knockdown A549 cells (data not shown). Although Bid is well known for proapoptosis signaling, many recent studies confirmed its role in early DDR (50, 56). Consistent with these studies, a transient exposure of cancer cells to CPT induced the expression of Bid at both transcript and protein levels in A549 (Figs. 5 A and data not shown) and H1299 cells (Fig. 5 B) and its nuclear localization (data not shown), suggesting its role in CPT-induced DDR.

To evaluate the role of Bid in CPT-induced DDR, we down-regulated Bid using siRNAs and assessed its role on CPT-induced Chk1 phosphorylation and clonogenic survival. Similar to Gli1 knockdown cells, Bid-deficient cells exhibited defects in CPT-induced phosphorylation of Chk1 in multiple cell lines (Fig. 5, D–F). Supporting its role in CPT-induced DDR, an average of a more than 2-fold increase in Bid protein levels was

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**Gli1 Regulates the ATR-mediated Replication Checkpoint by Controlling Bid Expression in Tumor Cells and Their Resistance to CPT**

Previous studies demonstrated no significant changes in the expression of genes involved in ATR-mediated Chk1 phosphorylation when Gli1 was overexpressed (25). Here we assessed whether down-regulation of Gli1 alters the expression of proteins involved in the ATR-Chk1 signaling cascade. A careful analysis of the protein levels in different cancer cells in this signaling cascade revealed no significant impact of Gli1 down-regulation. Further analysis of ATR and its downstream proteins Rad 9 (a protein in the 9-1-1 complex), Claspin, ATRIP, RPA, and Chk1, which are directly involved in the ATR-mediated checkpoint response, have not shown any significant differences compared with control cells (Figs. 2, A and B, and 5, A and B, and data not shown). These results directed us to search for the gene products reported to be involved in the ATR-mediated S-phase checkpoint leading to Chk1 phosphorylation.
observed in response to CPT (Fig. 5, A and B). Consistent with this, Bid levels directly correlated with phosphorylated Chk1 and Gli1. Similarly, Bid down-regulation also abrogated CPT-induced Chk1 phosphorylation (Fig. 5, D–F) and sensitized tumor cells to CPT (Fig. 5 C), implicating a novel role for Bid in CPT-induced DDR and cell resistance. Because the inhibition of aberrant Gli1 affects Bid expression and CPT-induced Chk1 phosphorylation in tumor cells (Fig. 5, A and B), these data suggest that Gli1 may regulate the S-phase checkpoint in these cells through the transcriptional regulation of Bid.

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**FIGURE 5.** Gli1 inhibition affects the S-phase checkpoint by down-regulation of Bid. A and B, Gli1-proficient and knockdown tumor cells were exposed to DMSO or 500 nM CPT and assessed for different DDR proteins involved in the ATR/Chk1 signaling cascade in A549 (A) and H1299 (B) cells. C–F, similarly, control and Bid down-regulated A549 cells were assessed for CPT sensitivity (C) and CPT-induced Chk1 phosphorylation in different cancer cells: A549 (D), H1299 (E) and A2780/CP70 (F). The data in C are mean ± S.D. of triplicates.

**Gli1 Down-regulation Affects the Association of ATR-ATRIP with RPA in Response to CPT, and Ectopic Expression of Bid in These Cells Complements Chk1 Phosphorylation**—Although conflicting results have been reported for the role for Bid in DDR (57, 58), the experimental data from Zinkel’s group demonstrated that Bid stimulates the association of ATRIP with RPA on chromatin in response to hydroxyurea and UV-induced replication stress (58). These studies have suggested a crucial role for Bid in maintaining a high-order structure of the DNA damage sensor complex important for ATR-mediated Chk1 phosphorylation. To assess whether Gli1 deficiency also affects the association between RPA and the ATRIP-ATR complex in response to CPT, we performed immunoprecipitation studies with RPA70 antibody and analyzed the levels of coimmunoprecipitated proteins in control and Gli1-depleted A549 cells. As shown in the protein input, levels of ATR, ATRIP, and RPA70 remained unaffected on the basis of the Gli1 status. Similarly, consistent with the role for Bid in replication stress-mediated checkpoint response (50), the CPT-induced association of ATRIP and ATR with RPA was affected drastically in Gli1 knockdown cells (Fig. 6A) compared with control siRNA-transfected cells. These data provide additional support for a role of Bid in replication stress-induced DDR and in promoting ATR-mediated Chk1 phosphorylation.

To test whether Gli1 controls Chk1 phosphorylation by regulating Bid expression in tumor cells, we reasoned that ectopic expression of Bid in Gli1 down-regulated cells should complement checkpoint defects. To examine this, we ectopically expressed Bid in Gli1 down-regulated A549 cells and assessed
the status of Chk1 phosphorylation. Remarkably, complementation of Bid by ectopic expression in Gli1 down-regulated cells restored Chk1 phosphorylation in response to CPT (Fig. 6B).

Therefore, our data further support the hypothesis that Gli1 influences ATR-mediated phosphorylation of Chk1 by regulating Bid in tumor cells.

**Gli1 Regulates Bid Promoter Activity in Luciferase Reporter Assays**—To assess whether Gli1-mediated regulation of Bid expression in tumor cells is through its promoter activity, we searched for consensus sites for Gli1 in the Bid promoter. Interestingly, a consensus site (GACCACCCCA) was identified within the 1.1 kb upstream of the transcription initiation codon (Fig. 7A). To test whether Gli1 status can influence gene expression through this 1-kb region, we transfected A549 cells with control or Gli1 siRNAs, and then these cells were cotransfected with either a Renilla luciferase expression vector under the control of the Bid promoter (1.1-kb upstream region) or a constitutive promoter. As shown in Fig. 7B, Gli1 knockdown cells demonstrated a more than 50% reduction in luciferase activity compared with Gli1-proficient cells. In contrast, Gli1 down-regulation had no significant effect on the luciferase activity of control (constitutive) luciferase vector-transfected cells. These data demonstrate that Gli1 regulates the promoter activity of Bid in tumor cells, probably through the consensus site (GACCACCCCA).

**Pharmacological Inhibition of Gli1 with GANT61 Abrogates Chk1 Phosphorylation and Induces DDR and Sensitizes Cancer Cells to CPT**—To validate the above siRNA-mediated inhibition of Gli1 signaling responses and to determine whether it is translatable, we used GANT61, a pharmacological inhibitor that has been reported to target both Glis (Gli1 and Gli2). Although GANT61 is used widely to inhibit Hh signaling at the Gli level, the mechanism by which GANT61 inhibits Gli1 and Gli2 and its specificity between the two Glis is not clear. However, similar to siRNA-mediated Gli1 down-regulation, GANT61 exposure to cancer cells also induced DDR (Fig. 8A), inhibited CPT-induced Chk1 phosphorylation, and potentiated its damage response, as evidenced by increased levels of \( \gamma H2AX \) (Fig. 8, B and D). Importantly, consistent with the Gli1 down-regulation-mediated cellular responses, pharmacological inhibition of Gli1 by GANT61 also exhibited down-regulation of Bid (Fig. 8, B and D) and sensitized cancer cells to CPT (Fig. 8C). Overall, our studies unraveled a novel role for aberrant Gli1 in tumor cells in the regulation of the replication checkpoint by ATR-mediated Chk1 phosphorylation via Bid transcriptional control to protect them from oncogenic replication stress and resistance to the therapeutics that target DNA replication (Fig. 9). Likewise, inhibition of Gli1 either by siRNAs or GANT61 hypersensitizes cancer cells to Top1 inhibitors.

**DISCUSSION**

Oncogene or growth signal-mediated replication stress is a common feature in cancer cells (59–61). It is expected that...
S-phase checkpoint signaling mediated by Chk1 plays a critical role in cell survival during replication stress and tumor resistance to chemotherapeutic agents (62–64). In this study, our results demonstrate that the abnormal expression of Gli1 in tumor cells regulates the S-phase checkpoint through ATR-mediated activation of Chk1. Similarly, inhibition of Gli1 in these tumor cells induces replication-mediated DNA damage. These observations suggest that aberrant expression of Hh signaling at the level of Gli1 promotes tumor progression and resistance to chemotherapy, at least partly but significantly, by regulating the S-phase checkpoint through Chk1-mediated signaling. Although Hh/Gli1 signaling is known to differentially regulate several genes involved in various cellular processes (65), the association of aberrant Gli1 with Chk1 phosphorylation in tumor cells implicates its role in suppression of replication stress in these tumors.

It is well accepted that oncogenes and altered growth signals force cells to enter into the cell cycle and that this induces replication stress. However, cell cycle checkpoints are coordinated events that are fine-tuned for the controlled and timely expression of different genes involved in relaying these signals. Previously, it has been reported that forced expression of Gli1 abrogates ionizing radiation-induced Chk1 phosphorylation and causes genomic instability in Patch+/−/H11001/H11002 mice and in Gli1-expressing HEK293 cells (25). In this study, we demonstrate that the inhibition of aberrant Gli1 in tumor cells abrogates the S-phase checkpoint. However, such differences in Hh/Gli1 signaling-mediated responses are well documented. For example, it has been shown that Gli1 expression alone is sufficient for the development of medulloblastoma and spontaneously or radiation-induced skin carcinogenesis (5, 66–68). On the other hand, a tumor suppressor role has been attributed to Gli1 in neuroblastoma development (69, 70). These distinct responses could be attributed to the tissue type, the cellular context, and the signaling mechanisms that activate Hh/Gli1 expression (9, 71). Importantly, this study demonstrates that these spontaneous DDRs in Gli1-depleted tumor cells are replication stress-mediated events, as evidenced by most of the γH2AX foci colocalizing with EdU in these cells (Fig. 4C). Because the
replication checkpoint mediated by Chk1 is important for fork stability and proper progression through the cell cycle, the spontaneous DDR in Gli1-depleted cells could be readily attributed to a deficiency in the S-phase checkpoint. Similar results have also been observed in several independent studies when Chk1 was inhibited in cancer cells (39, 72, 73). We and others have shown previously that Top1 poisoning by CPT not only induces replication stress but also leads to replication-coupled DSBs because prolonged stabilization of Top1-cc by CPT can lead to fork collapse (29, 31, 37, 72, 73). Therefore, active replication is important for CPT-induced cytotoxic effects. In response to Top1 inhibitors, Chk1 inhibits DNA replication at both initiation and elongation to minimize CPT-induced DNA damage (33). In the absence of Chk1 signaling, it is expected that the probability of replication fork collision with Top1-cc increases and, therefore, maximizes the cytotoxic effects of Top1 inhibitors (36). Consistent with the observations in Chk1-depleted or inhibited cells, Gli1 inhibition abrogated Chk1 phosphorylation in response to CPT, enhanced γH2AX foci, and sensitized cancer cells to Top1 inhibition in several cancer cells. These observations are consistent with a model in which CPT-induced cytotoxicity is caused by the generation of replication-coupled DSBs (32, 74).

Several studies have implicated Bid in mediating DDR signaling (50, 58, 75). Moreover, recent mechanistic studies have demonstrated a critical role for Bid in the association of RPA with the ATRIP-ATR complex in response to hydroxyurea (HU)- and UV-induced replication stress (50) and sensitivities of BID−/− MEFs (mouse embryonic fibroblasts) to several chemotherapeutic agents (75). Consistent with these studies, our data further support a Bid role in ATR-mediated Chk1 phosphorylation in response to the Top1 poison CPT. Importantly, our studies establish a novel regulatory mechanism of Bid in cancer cells by aberrant Gli1 and its role in CPT-induced replication checkpoint signaling. Moreover, the absence of Gli1 expression in normal cells and the Gli1 inhibition-mediated abrogation of the S-phase checkpoint in tumor cells give a potential therapeutic strategy where the damage to normal cells (bystander effect) can be minimized by CPT treatment. Therefore, our studies suggest that hedgehog signaling inhibitors at the level of Gli1 may contribute to effective treatment options specifically in combination with Top1 inhibitors.

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