SUPPLEMENTARY MATERIALS AND METHODS:

Bivariate Flow Cytometric Analysis: Bivariate flow cytometric analysis determines the expression of proteins simultaneously with measurement of DNA content and phase of the cell cycle (1). HT29 cells were plated at a density of 100,000 cells/well in 6-well plates and allowed to attach overnight. Media was removed and cells were treated, in duplicate, in the absence or presence of GANT61 (20μM) or cyclopamine (20μM) for up to 40 hr. Cells were harvested, and staining performed as described (1) using primary antibodies for $p21^{Cip1}$, cyclin E or cyclin A, and fluorescein (FITC)-labeled goat anti-rabbit or anti-mouse secondary antibodies. Cells were washed, resuspended in buffer containing PBS, propidium iodide (5 μg/ml) and DNase-free RNase A (200 μg/ml), and samples were analyzed by flow cytometry according to established procedures for cell cycle distribution studies (1).

Gene knockdown using shRNA:

$p21^{Cip1}$shRNA: (5'-CGACTTTGTCACCGAGACACCACTGGAGG-3') and scrambled shRNA, were obtained from OriGene Technologies, Inc, (MD). HT29 cells stably expressing $p21^{Cip1}$shRNA or scrambled shRNA (control) were generated by transducing HT29 cells with shRNA expressing retroviruses for 48 hr (50% retroviral supernatant and 50% normal growth media) in the presence of polybrene (Sigma-Aldrich, MO; 4 μg/ml final concentration). Following transduction the cells were washed x 3 with PBS and allowed to grow for 3 passages before screening for gene expression. Stable expression of $p21^{Cip1}$shRNA was ensured by culturing cells in the presence of the selection antibiotic, puromycin (1 μg/ml). Once decreased expression of $p21^{Cip1}$ was confirmed by western analysis, the cells were used for experiments.

COMET Assay: HT29 cells (1 x 10^6/10 cm plate) were treated with GANT61 (20 μM), cyclopamine (20 μM) or vehicle alone (0.2% DMSO) and incubated at 37°C in a 5% CO_2 incubator for up to 48 hr. Positive control cells were generated by treating cells with H_2O_2 (100 μM) for 20 min in the dark at 4°C. Cells were harvested, processed, and electrophoresed in agarose gels as described (2).

Microscopy: For confocal microscopy, cells were plated at a density of 50,000/well in 6-well plates on coverslips and allowed to attach overnight. The following day, media was removed and the cells were treated in the absence or presence of GANT61 (20μM) or cyclopamine (20μM) for up to 48 hr. The coverslips were removed and placed in a humidity chamber for fixation, permeabilization and staining. Images were collected using an HCX PI Apo 63X, 1.4NA oil immersion objective with Zoom 2 on a Leica SP2 confocal microscope with spectrophotometric detection (Leica Microsystems, GmbH, Wetzlar, Germany). Four-color image acquisition was performed with the following Excitation/Emission settings: 364nm Ex/400-480nm Em, 488nm Ex/500-550nm Em, 561nm Ex/570-630nm Em, 633nm Ex/640-750nm Em. Post-processing and analysis of images was conducted using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). For live cell image capturing, the cells were treated with GANT61 or transfected with Gli3R for 72 hr followed by microscopy. The images were collected using an inverted microscope (Leica Microsystems, GmbH, Wetzlar, Germany) equipped with Retiga SRV cooled CCD camera with Liquid Crystal tunable RGB filter (QImaging, Surrey, BC Canada).

ChIP analysis: HT29 cells treated with GANT61 (20 μM) for 24 hr were cross-linked in

1% formaldehyde/PBS, 10 min, 37^{o}C, which was terminated in glycine, 5 min. Cells were washed in PBS and nuclear extracts prepared. Nuclei were sonicated to generate chromatin fragments (≈ 500 to 800 bp); 30 µl were treated with RNase A and Proteinase K followed by de-cross-linking and DNA isolation. The chromatin was precleared with a mixture of proteinA-sepharose and proteinG-sepharose that was blocked with bovine serum albumin (1 mg/ml) and Salmon sperm DNA (1 mg/ml). 10% of the precleared chromatin was used as input control. Equal quantities of the precleared chromatin fragments were immunoprecipitated with antibodies specific for Gli1 (Novus Biologicals, CO), Gli2 (Cell Signaling Technology (MA), IgG (Abcam, MA; negative control), or histone H3 (Abcam, MA; positive control). Methods were performed as described previously (3, 4). Subsequent qRT-PCR employed GFAP primers that flanked the promoter regions of the GLI target genes HIP1, BCL-2 and CCND2, or FAS (negative control). PCR products were resolved on a 1% agarose gel and stained with ethidium bromide to visualize under uv light.

Luciferase reporter assays: HT29 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with 4 µg of Gli-luc or NF-κB-luc or AP1-luc and 0.4 µg of pRLTK (Renilla Luciferase driven by TK promoter). Twenty-four hr post-transfection, cells were treated with GANT61 (20 µM) for 24 hr, and harvested using the dual luciferase kit (Promega Corporation, WI) according to the manufacturer's protocol. Luciferase activity was detected by a Victor2 multilabel counter, and normalized to renilla luciferase activity as a control for transfection efficiency.

SUPPLEMENTARY REFERENCES:

1. Juan G, Darzynkiewicz Z. Bivariate analysis of DNA content and expression of cyclin proteins. Curr Protoc Cytom 2001;Chapter 7:Unit 7 9.
2. Plesca D, Crosby ME, Gupta D, Almasan A. E2F4 function in G2: maintaining G2-arrest to prevent mitotic entry with damaged DNA. Cell Cycle 2007;6:1147-52.
3. Weinmann AS, Farnham PJ. Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation. Methods (San Diego, Calif 2002;26:37-47.
4. Wells J, Farnham PJ. Characterizing transcription factor binding sites using formaldehyde crosslinking and immunoprecipitation. Methods (San Diego, Calif 2002;26:48-56.

SUPPLEMENTARY FIGURE LEGENDS:

Supplementary Figure 1. BrdU incorporation demonstrates accumulation of GANT61-treated cells at the G1/S boundary and in early S. HT29 cells were treated with GANT61 (20 μM) or cyclopamine (20 μM) for up to 48 hr. % BrdU incorporation was determined using flow cytometry. Data are representative of duplicate determinations.

Supplementary Figure 2. Representative scatter plot of cyclin A expression vs. DNA content distribution in HT29 cells treated with either GANT61 (20 μM; left panel) or cyclopamine (20 μM; right panel).