cDNA Microarray Gene Expression Profiling of Hedgehog Signaling Pathway Inhibition in Human Colon Cancer Cells

Ting Shi¹, Tapati Mazumdar¹, Jennifer DeVechio¹, Zhong-Hui Duan², Akwasi Agyeman¹, Mohammad Aziz¹*, Janet A. Houghton¹*

¹ Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio, United States of America, ² Department of Computer Science, University of Akron, Akron, Ohio, United States of America

Abstract

Background: Hedgehog (HH) signaling plays a critical role in normal cellular processes, in normal mammalian gastrointestinal development and differentiation, and in oncogenesis and maintenance of the malignant phenotype in a variety of human cancers. Increasing evidence further implicates the involvement of HH signaling in oncogenesis and metastatic behavior of colon cancers. However, genomic approaches to elucidate the role of HH signaling in cancers in general are lacking, and data derived on HH signaling in colon cancer is extremely limited.

Methodology/Principal Findings: To identify unique downstream targets of the GLI genes, the transcriptional regulators of HH signaling, in the context of colon carcinoma, we employed a small molecule inhibitor of both GLI1 and GLI2, GANT61, in two human colon cancer cell lines, HT29 and GC3/c1. Cell cycle analysis demonstrated accumulation of GANT61-treated cells at the G1/S boundary. cDNA microarray gene expression profiling of 18,401 genes identified Differentially Expressed Genes (DEGs) both common and unique to HT29 and GC3/c1. Analyses using GenomeStudio (statistics), Matlab (heat map), Ingenuity (canonical pathway analysis), or by qRT-PCR, identified p21<sup>WAF1/CIP1</sup> (CDKN1A) and p15<sup>INK4b</sup> (CDKN2B), which play a role in the G1/S checkpoint, as up-regulated genes at the G1/S boundary. Genes that determine further cell cycle progression at G1/S including E2F2, CYCLIN E2 (CCNE2), CDC25A and CDK2, and genes that regulate passage of cells through G2/M (CYCLIN A2 [CCNA2], CDC25C, CYCLIN B2 [CCNB2], CDC20 and CDC2 [CDK1]), were down-regulated. In addition, novel genes involved in stress response, DNA damage response, DNA replication and DNA repair were identified following inhibition of HH signaling.

Conclusions/Significance: This study identifies genes that are involved in HH-dependent cellular proliferation in colon cancer cells, and following its inhibition, genes that regulate cell cycle progression and events downstream of the G1/S boundary.

Introduction

Hedgehog (HH) signaling plays a critical role in a variety of normal cellular processes. It is pivotal in embryogenesis, regulation of the epithelial-to-mesenchymal transition, the patterning of a diverse range of vertebrate structures in a variety of organs, maintenance of adult tissue homeostasis, tissue repair, cellular proliferation, and in cell survival [1,2,3,4,5,6,7,8,9]. The canonical HH pathway is also critical to normal mammalian gastrointestinal development, where it is involved in the coordinate regulation of differentiation of normal intestinal villi [10,11,12]. Thus, in the normal gastrointestinal tract, HH ligands are induced in the differentiated cells around the villous surface, generating a negative feedback loop to inhibit canonical WNT signaling in the basal cells of the crypt, thereby protecting differentiated cells from the proliferative effects of WNT [13]. Activation of the canonical HH signaling pathway comprises the binding of HH ligands to the membrane receptor Patched (PTCH1), which becomes internalized leading to the activation of the signaling molecule Smoothened (SMO) via release from PTC-mediated suppression. SMO activates the final arbiter of HH signaling, the GLI family of transcription factors that bind to the GAC-CACCCA-like consensus binding element in promoter sequences to transcriptionally regulate HH target genes [3,14,15]. GLI1 and GLI2, the transcriptional activators of HH signaling, possess distinct as well as overlapping functions that involve activator (GLI1 and GLI2) or repressor (GLI2) activities [16]; however, their roles in the regulation of HH-driven cellular proliferation,
survival or cell death processes are poorly understood. Historically, GLI1 has been considered the most reliable marker of HH pathway activity, however GLI2 appears to be the primary activator of HH signaling, with GLI1 as a transcriptional target of GLI2 [3,7], leading to augmentation of HH signaling both quantitatively as well as qualitatively [16]. An important feature of GLI proteins is that their biological activity is context-dependent, influenced by the cellular environment [17,18].

Activation of the canonical HH signaling cascade is aberrantly activated and well known to play a critical role in oncogenesis and maintenance of the malignant phenotype in several types of human cancers. Such activation involves amplification of GLI1 or GLI2, mutations in PTC or SMO, or dysregulated gene expression [3,4]; these malignant cells are also sensitive to the small molecule inhibitor that targets SMO, cyclopamine [4,19,20,21,22,23]. Colon carcinomas are thought to derive from constitutive activation of WNT signaling by mutation of the APC or ß-CATENIN genes, while the involvement of the HH signaling pathway is not as clear. In gastrointestinal malignancies, transcriptional up-regulation of HH ligands has been identified as the predominant activator of HH signaling in these diseases (reviewed in [3]). In addition, there is emerging evidence that HH signaling is involved in colorectal carcinogenesis [24,25], colon carcinoma stem cell self renewal, and in the metastatic behavior of advanced colon cancers [26]. However, genomic approaches to elucidate the role of HH signaling in cancers in general are lacking, regulatory genes downstream of GLI1 and GLI2 that function in cellular proliferation, survival, and maintenance of the malignant HH phenotype remain incompletely characterized [5], and data derived on HH signaling in colon cancer is extremely limited.

Cellular proliferation is driven by progression of cells through the cell cycle consisting of sequential passage through G1, S, G2 and M phases. Cyclin-dependent kinases (CDKs) associate with cyclins to drive the cell cycle machinery [27,28]. Thus, CDK2 associates with CYCLIN E at the G1/S transition and with CYCLIN A during S-phase, CDK4 and CDK6 bind to CYCLIN D during progression at G1/S, while CDC2 complexes with CYCLIN A at G2, and with CYCLIN B during the G2/M transition. CDC25 family members also regulate cell cycle progression through dephosphorylation of the CDKs [29,30]. CDK inhibitors, including p21Cip1 [29,31] and p15Ink4B [32], bind to cyclin-CDK complexes during the cell cycle transition, in particular at G1/S (p21Cip1, p15Ink4B [32] and G2/M (p21Cip1, [29]), and can also induce cell cycle arrest at the G1/S boundary following cytostatic signals through functional inhibition of cyclin-CDK complexes. The E2F family of transcription factors also regulates the expression of genes required for the G1/S transition, in particular genes involved in the activation of the DNA replication machinery, and DNA repair [33].

cDNA microarray technology has provided the ability to study the expression of thousands of genes simultaneously, and is an important tool in the dissection of signal transduction pathways. For the HH signaling cascade, HH/GLI target gene expression has been examined following EGF stimulation [6] or inducible GLI1 [16] or GLI2 [9] gene activation in human keratinocytes, or in GLI1-induced cell transformation [7]. To identify unique downstream targets of the GLI genes that function in cellular proliferation in the context of colon carcinoma, we employed a small molecule inhibitor of both GLI1 and GLI2, GANT61, identified in a cell-based small molecule screen for inhibitors of GLI1-mediated transcription [34]. GANT61 acts in the nucleus to block GLI1 function, inhibits both GLI1- and GLI2-mediated transcription, and demonstrates a high degree of selectivity for HH/GLI signaling [34]. Thus, GANT61 acts downstream of cyclopamine (targeting SMO) to inhibit the final determinants of HH transcriptional regulation.

In two human colon carcinoma cell lines, HT29 and GC3/c1, inhibiting the HH signaling pathway using GANT61 decreased expression of GLI1, GLI2 and the HH ligand receptor, PTC1, and inhibited proliferation by inducing cellular accumulation at the G1/S boundary 24 hr after treatment, determined by flow cytometric analysis. On further detailed analysis using cDNA microarray gene expression profiling and quantitative Real-Time PCR, p21Cip1 (CDKN1A) and p15Ink4B (CDKN2B), that can elicit the G1/S checkpoint, were up-regulated, while genes that further determine entry from G1 to S-phase including E2F2, CYCLIN E2 (CCNE2), CDC25A and CDK2 were decreased in expression. Concomitant with decreased G1 to S-phase progression, decreased expression of CYCLIN A2 (CCNA2), CDC25C, CYCLIN B2 (CCNB2), CDC20 and CDC2 (CDK1), that regulate the passage of cells through G2/M were also demonstrated. Additional novel genes that are involved in stress response, and the response to DNA damage, not previously identified following termination of HH signaling in human cancer cells, include the early response genes DDT2 (GADD45G), DDT3 (GADD153), DDT4 (REDD1), PPIP15A (GADD34) and ATF3 that were significantly up-regulated. Genes involved in DNA synthesis and repair (TYMS, TOP2A, TK1, POLE, POLE2), and additional novel genes involved in S-phase progression or DNA damage responses that were significantly down-regulated, include KIAA0101 (p15 [PAF]), Replication Factor C variants 2, 3, 4, 5, CDT1, the E2F transcription factors CDC4A and TFDP1, MDC1, FANCDC, PCNA, and the genes involved in DNA repair, RAD51C (XRCC3), RAD54B, RAD51 and HELLs. This study has therefore identified genes that are regulated during the termination of HH-dependent cellular proliferation and survival in colon cancer cells, and involves genes associated with G1/S-phase arrest, DNA damage and stress responses.

Results

GANT61 induces down-regulated expression of GLI genes and accumulation of human colon carcinoma cells at G1/S

In HT29 and GC3/c1 cells treated with GANT61 (20 μM) for up to 48 hr, expression of the target genes GLI1 and GLI2 were both down-regulated, and also the HH ligand receptor PTC1, as determined by qRT-PCR (Figure 1A). Subsequently, HT29 or GC3/c1 cells were treated, in duplicate, with GANT61 (20 μM) followed by PI staining and flow cytometric analysis for the determination of cell cycle distribution between G1, S and G2/M phases (Figure 1B). In both cell lines, cells accumulated in G1, 24 hr after treatment with GANT61. In GANT61-treated HT29 cells, a 20% increase in G1-phase cells was associated with a corresponding decrease in cells within the G2/M phase (14%) and in S-phase (5%), consistent with a G1/S checkpoint arrest. In GC3/c1 cells, an 8% increase in G1-phase cells at 24 hr after GANT61 treatment also corresponded with a similar reduction in cells in the G2/M phase of the cell cycle (Figure 1B).

cDNA microarray analyses identify changes in gene expression both common and unique to HT29 and GC3/c1 following GANT61 treatment

To delineate the changes in gene expression in HT29 and GC3/c1 human colon carcinoma cell lines in response to treatment with the GLI1/GLI2 antagonist, GANT61, the
Figure 1. Expression of GLI1, GLI2, PTCH1 and cell cycle distribution of HT29 and GC3/c1 cells. Cells were treated for up to 48 hr with GANT61 (20 μM) or with 0.2% DMSO (vehicle control). (A) qRT-PCR of GLI1, GLI2 and PTCH1 genes from time 0 to 48 hr. (B) DNA was extracted at 24 hr after treatment, stained with propidium iodide, and subsequently analyzed for the effects of inhibition of HH signaling on phase distribution of cells within the cell cycle by flow cytometry.

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expression of 18,401 human genes was profiled in control cells treated with vehicle (0.2% DMSO) and in cells treated with GANT61 (20 μM) for 24 hr. Genes with a False Discovery Rate (FDR)-adjusted p-value of <0.001 and fold change >1.5 were considered Differentially Expressed Genes (DEGs) induced by GANT61 relative to the vehicle control, of which 1,368 genes were differentially expressed in HT29, and 1,002 genes in GC3/c1 cells (Figure 2). 755 genes or 338 genes were up-regulated, and 613 or 444 genes were down-regulated, in HT29 and GC3/c1 cells, respectively. 763 and 397 genes were differentially expressed and unique to HT29 or GC3/c1, respectively. Of the 763 DEGs unique to HT29, 459 (60%) were up-regulated and 304 (40%) were down-regulated. Similarly, of the 397 DEGs unique to GC3/c1, 262 (66%) were up-regulated and 135 (34%), down-regulated. In contrast, 605 genes representing 3.4% of all genes were unique to HT29, 459 (60%) were up-regulated and 304 (40%) were down-regulated. Similarly, of the 397 DEGs unique to GC3/c1, 262 (66%) were up-regulated and 135 (34%), down-regulated. In total, 603 genes representing 3.4% of all genes were differentially expressed that were common to both cell lines; of these, 296 were up-regulated (49%), and 309 (51%) were down-regulated (Figure 2). All genes common to both HT29 and GC3/c1 that were significantly up-regulated or down-regulated (p<0.001) are listed in Tables 1 and 2, respectively.

Modulation of canonical signaling pathways following inhibition of HH signaling

Genes with significant changes in expression following GANT61 treatment were assigned to different canonical signaling pathways and subjected to Ingenuity Pathway Analysis (IPA), where the resulting 1,368 DEGs in HT29 and 1,002 DEGs in GC3/c1 were mapped to networks defined by the IPA database (Figure 3). For the mapped DEGs including both up- and down-regulated genes, the 15 most significantly altered canonical pathways in HT29 demonstrated –log(p-value) ranging from 2.045 to 9.025, and in GC3/c1 from 2.32 to 7.509. Of the 15 pathways involving genes significantly down-regulated, 12 were common to both cell lines. The 3 common pathways with the greatest differential down-regulated expression include genes involved in the DNA damage response, cell cycle checkpoint, and mitosis. Other pathways down-regulated involved the G1/S and G2/M DNA damage checkpoints, DNA precursor metabolism, and cell signaling involving different pathways including those involved in cancers, which also demonstrate 3 signatures unique to either HT29 or GC3/c1 (Figure 3). Of the 15 pathways, involving genes that are the most significantly up-regulated, 8 are common to both HT29 and GC3/c1, and 7 represent unique pathways for each cell line, demonstrating more diversity in patterns of up-regulated gene expression (Figure 3). The up-regulated pathways common to both cell lines include the metabolism-related such as steroids, pyruvate, glycolysis glutathione, or glycerolipid, and not directly related to the control of cellular proliferation.

Genes that demonstrate differential fold change patterns in GANT61-treated HT29 and GC3/c1 cells

Fold change patterns of most highly DEGs in HT29 and GC3/c1 were selected, analyzed and displayed in a heat map to evaluate and compare similarity and differences in differential expression between the two cell lines treated with GANT61 (Figure 4). In addition to genes with diverse functions that are not directly related to HH-dependent proliferation, up-regulated genes that influence the G1/S transition and subsequent cell cycle progression, and that are common to both cell lines, include CDKN1A, and the DNA-damage-inducible transcripts 3 and 4 (DDIT3 and DDIT4). A considerably greater number of genes involved in cellular proliferation and cell cycle transition through the G1/S boundary, S-phase progression, and the G2/M transition, were significantly down-regulated in expression, and common to both cell lines. These include CDC6 (involved at G1/S), three genes that drive entry into and passage through S-phase (CCNE2, E2F2) and G2 (CCNA2), genes involved in DNA replication and repair (TYMS, POLE, TOP2A, TK1, POLE2), and two genes that regulate mitosis (AURKB, CDC20; Figure 4, asterisks). Of the 296 up-regulated genes, in addition to the genes comparably represented in the heat map that include DDIT3 (GADD153) and DDIT4 (REDD1), additional novel DNA damage-inducible transcripts were also identified and include DDIT2 (GADD45G), PPP1R15A (GADD34) and ATF3 (Table 1). TP53INP1, which can regulate cell cycle arrest, and TP53INP2, identified in cell death responses, were also up-regulated. Of the 309 genes significantly down-regulated in response to GANT61, novel genes identified include KIAA0101 (p15[PAF]), Replication Factor C variants 2, 3, 4, 5, CDT1, the E2F transcription factors CDC44 and TDFP1, MDC1, PCNA, FANCD2, and the genes involved in DNA repair, RAD51C (XRCC3), RAD54B, RAD51 and HELLS (Table 2).

Differentially expressed genes involved in the G1/S and G2/M transitions

To further evaluate the genes involved in control of cell cycle progression in human colon carcinoma cells following GANT61 treatment, 10 genes involved in the G1/S or G2/M transitions, identified by IPA, were selected for further examination. Genes required at the G1/S boundary for G1/S transition, or for the induction of a G1/S checkpoint following cytostatic signals,
| ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change |
|------------------|-------------|------------|-------------|
| NM_004893.3      | SDCBP2      | syndecan binding protein | 12.42 10.83 |
| NM_001042483.1   | NUPR1       | nuclear protein 1 | 7.96 3.53 |
| NM_021187.2      | CYP4F11     | cytochrome P450 | 7.73 4.27 |
| NM_001001870.1   | C17orf91    | chromosome 17 open reading frame 91 | 6.79 3.82 |
| NM_004864.1      | GDF15       | growth differentiation factor 15 | 6.61 3.43 |
| NM_004083.4      | DDIT3       | DNA-damage-inducible transcript 3 | 6.36 2.35 |
| NM_004083.4      | DDIT3       | DNA-damage-inducible transcript 3 | 6.36 2.35 |
| NM_001008213.1   | OPTN        | optineurin | 7.96 3.53 |
| NM_004864.1      | GDF15       | growth differentiation factor 15 | 6.61 3.43 |
| NM_004083.4      | DDIT3       | DNA-damage-inducible transcript 3 | 6.36 2.35 |
| NM_004083.4      | DDIT3       | DNA-damage-inducible transcript 3 | 6.36 2.35 |
| NM_001008213.1   | OPTN        | optineurin | 7.96 3.53 |
| NM_004864.1      | GDF15       | growth differentiation factor 15 | 6.61 3.43 |
| NM_004083.4      | DDIT3       | DNA-damage-inducible transcript 3 | 6.36 2.35 |
| NM_004083.4      | DDIT3       | DNA-damage-inducible transcript 3 | 6.36 2.35 |
| NM_001008213.1   | OPTN        | optineurin | 7.96 3.53 |
| NM_004864.1      | GDF15       | growth differentiation factor 15 | 6.61 3.43 |
| NM_004083.4      | DDIT3       | DNA-damage-inducible transcript 3 | 6.36 2.35 |

**Table 1.** Up-regulated genes (p<0.001) common in GANT61-treated HT29 and GC3/c1 cells.
| ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change | ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change |
|------------------|-------------|------------|-------------|------------------|-------------|------------|-------------|
| ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change | ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change |
| HT29 GC3/c1      | HT29 GC3/c1 | HT29 GC3/c1 | HT29 GC3/c1 |
| NM_006918.4      | SCSDL       | sterol-CS-desaturase | 3.68 | 3.41 | NM_002421.2 | CLIP2       | CAP-GLY domain containing linker protein 2 | 2.27 | 1.79 |
| NM_182980.2      | OSGIN1      | oxidative stress induced growth inhibitor 1 | 3.55 | 4.48 | NM_014039.2 | C11orf54    | chromosome 11 open reading frame 54 | 2.27 | 1.62 |
| NM_025182.2      | KIAA1539    | KIAA1539 | 3.49 | 2.62 | NM_017585.2 | SLC2A6      | solute carrier family 2 | 2.27 | 2.35 |
| NM_018677.2      | ACS52       | acyl-CoA synthetase short-chain family member 2 | 3.46 | 4.68 | NM_014330.2 | PPP1R15A    | protein phosphatase 1 | 2.26 | 1.63 |
| NM_001034850.1   | FAM134B     | family with sequence similarity 134 | 3.44 | 1.90 | NM_145306.2 | C10orf35    | chromosome 10 open reading frame 35 | 2.26 | 2.19 |
| NM_153742.3      | CTH         | cystathionase (cystathionine gamma-lyase) | 3.38 | 2.13 | NM_003831.3 | RIOK3       | RIO kinase 3 | 2.25 | 1.93 |
| NM_021158.3      | TRIB3       | tribbles homolog 3 | 3.34 | 3.71 | NM_006997.2 | TACC2       | transforming, acidic coiled-coil containing protein 2 | 2.25 | 1.95 |
| NM_000434.2      | NEU1        | sialidase 1 | 3.28 | 2.00 | NM_001006932.1 | RPS6KA2 | ribosomal protein S6 kinase | 2.24 | 1.68 |
| NM_01005404.3    | YPEL2       | yippee-like 2 | 3.22 | 2.40 | NM_000574.2 | CD55        | CD55 molecule | 2.23 | 1.84 |
| NM_004508.2      | ID1         | isopentenyl-diphosphate delta isomerase 1 | 3.21 | 4.78 | NM_018310.2 | BRF2        | subunit of RNA polymerase III transcription initiation factor | 2.23 | 2.14 |
| NM_003151.2      | STAT4       | signal transducer and activator of transcription 4 | 3.18 | 3.85 | NM_001491.2 | GCNT2       | glucosaminyl (N-acetyl)transferase 2 | 2.22 | 4.08 |
| NM_005165.2      | ALDOC       | aldolase C | 3.16 | 2.44 | NM_003567.2 | BCA3        | breast cancer anti-estrogen resistance 3 | 2.21 | 1.79 |
| NM_014331.3      | SLC7A11     | solute carrier family 7 | 3.16 | 2.54 | NM_005488.1 | TOM1        | target of myb1 | 2.20 | 1.89 |
| NM_000104.2      | CYP1B1      | cytochrome P450 | 3.15 | 2.41 | NM_004462.3 | FDF1T       | farnesyl-diphosphate farnesyltransferase 1 | 2.19 | 2.73 |
| NM_002340.3      | LSS         | lanosterol synthase | 3.14 | 3.15 | NM_019116.2 | UFD1        | ubiquitin family domain containing 1 | 2.19 | 1.90 |
| NM_078487.2      | CDKN2B      | cyclin-dependent kinase inhibitor 2B | 3.13 | 2.13 | NM_007105.1 | SLC22A18AS | solute carrier family 22 | 2.18 | 1.81 |
| NM_002201.4      | ISG20       | interferon stimulated exonuclease gene 20kDa | 3.12 | 1.91 | NM_182491.9 | ZFAND2A     | zinc finger | 2.17 | 2.17 |
| NM_016315.2      | GULP1       | GULP, engulfment adaptor PTB domain containing 1 | 3.11 | 4.39 | NM_181785.2 | SLC46A3     | solute carrier family 46 | 2.17 | 2.00 |
| NM_0028434.1     | WIP1        | WD repeat domain | 3.09 | 2.40 | NM_024866.4 | ADM2        | adrenomedullin 2 | 2.16 | 1.77 |
| NM_021465.1      | PHF1        | PHD finger protein 1 | 3.07 | 2.42 | NM_021133.2 | RNASEL      | ribonuclease L | 2.15 | 2.28 |
| NM_001995.2      | ACSL1       | acyl-CoA synthetase long-chain family member 1 | 3.06 | 2.86 | NM_032548.2 | ABTB1       | ankyrin repeat and BTB (POZ) domain containing 1 | 2.14 | 1.58 |
| NM_004354.1      | CCNG2       | cyclin G2 | 3.05 | 2.64 | NM_002153.1 | HSD17B2     | hydroxysteroid (17-beta) dehydrogenase 2 | 2.14 | 2.98 |
| NM_002073.2      | CCPG1       | cell cycle progression 1 | 3.04 | 1.88 | NM_006033.2 | LIPG        | lipase | 2.13 | 2.86 |
| NM_001017369.1   | SC4MOL      | sterol-C4-methyl oxidase-like | 3.03 | 4.04 | NM_020919.2 | ALS2        | amyotrophic lateral sclerosis 2 | 2.11 | 1.77 |
| NM_001031744.1   | LOC158160   | hypothetical protein | 3.02 | 2.35 | NM_014701.2 | KIAA0256    | KIAA0256 gene product | 2.11 | 2.30 |
| NM_032409.2      | PINK1       | PTEN induced putative kinase 1 | 2.98 | 2.44 | NM_001222.2 | CAMK2G      | calcium/calmodulin-dependent protein kinase | 2.11 | 2.12 |
| NM_003811.2      | TNFSF9      | tumor necrosis factor (ligand) superfamily | 2.98 | 3.04 | NM_015271.2 | TRIM2       | tripartite motif-containing 2 | 2.11 | 1.79 |
| NM_002357.2      | MXD1        | MAX dimerization protein 1 | 2.93 | 2.30 | NM_174936.2 | PCSK9       | proprotein convertase subtilisin/kexin type 9 | 2.11 | 4.18 |
| NM_000777.2      | CYP3A5      | cytochrome P450 | 2.92 | 1.77 | NM_015713.3 | RRM2B       | ribonucleotide reductase M2 B | 2.10 | 1.66 |
| NM_001098500.1   | KIAA1217    | KIAA1217 | 2.88 | 3.16 | NM_005749.2 | TOB1        | transducer of ERBB2 | 2.09 | 1.58 |
| NM_022119.3      | PRSS22      | protease | 2.84 | 3.27 | NM_004419.3 | DUSP5       | dual specificity phosphatase 5 | 2.08 | 3.61 |
| NM_004669.2      | CLIC3       | Homo sapiens chloride intracellular channel 3 | 2.80 | 6.19 | NM_033028.2 | BBS4        | Bardet-Biedl syndrome 4 | 2.08 | 2.04 |
| NM_080491.1      | GAB2        | GRB2-associated binding protein 2 | 2.79 | 2.06 | NM_001286.2 | CLCN6       | chloride channel 6 | 2.07 | 2.18 |
| ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change | ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change |
|------------------|-------------|------------|-------------|------------------|-------------|------------|-------------|
| NM_178270.1      | ATG4A       | ATG4 autophagy related 4 homolog A | 2.79 1.92 | NM_001132495.1  | SLC26A11    | PREDICTED:solute carrier family 26 | 2.07 1.87 |
| NM_002770.2      | PRSS2       | protease, serine, 2 (trypsin 2) | 2.79 1.64 | NM_172070.2     | ZNF650      | zinc finger protein 650 | 2.07 1.90 |
| NM_182608.2      | ANKRD33     | ankyrin repeat domain 33 | 2.74 1.91 | NM_004567.2     | PFKFB4      | 6-phosphofructo-2-kinase/fructose-2 | 2.07 1.93 |
| NM_006705.2      | GADD45G     | growth arrest and DNA-damage-inducible | 2.74 3.01 | NM_002555.3     | SLC22A18    | solute carrier family 22 | 2.06 1.62 |
| NM_012326.2      | MAPRE3      | microtubule-associated protein | 2.73 2.16 | NM_001010990.1  | HERPUD1     | homocysteine-inducible, ubiquitin-like domain member 1 | 2.06 1.63 |
| NM_001013251.1   | SLC3A2      | solute carrier family 3 | 2.72 2.40 | NM_198867.1     | ALKBH6      | alkB, alkalization repair homolog 6 | 2.06 1.65 |
| NM_016243.2      | CYB5R1      | cytochrome b5 reductase | 2.72 2.52 | NM_000271.3     | NPC1        | Niemann-Pick disease | 2.05 2.18 |
| NM_198129.1      | LAMA3       | laminin | 2.65 3.36 | NM_006454.2     | MXD4        | MAX dimerization protein 4 | 2.05 1.80 |
| NM_000228.2      | LAMB3       | laminin | 2.65 2.27 | NM_021194.2     | SLC30A1     | solute carrier family 30 | 2.03 1.73 |
| NM_006608.1      | PHTF1       | putative homeodomain transcription factor 1 | 2.64 2.38 | NM_173843.1     | IL1RN       | interleukin 1 receptor antagonist | 2.03 10.01 |
| NM_021229.3      | NTN4        | netrin 4 | 2.03 2.47 | NM_152271.1     | LONRF1      | LON peptidase N-terminal domain and ring finger 1 | 1.72 3.04 |
| NM_002064.1      | GLRX        | glutaredoxin | 2.03 1.75 | NM_003314.1     | TTC1        | tetratricopeptide repeat domain 1 | 1.72 1.55 |
| NM_005896.2      | IDH1        | isocitrate dehydrogenase 1 | 2.02 2.37 | NM_005920.2     | MEF2D       | myocyte enhancer factor 2D | 1.71 1.85 |
| NM_144498.1      | OSBPL2      | oxysterol binding protein-like 2 | 2.01 1.81 | NM_016410.2     | CHMP5       | chromatin modifying protein 5 | 1.71 2.14 |
| NM_005155.3      | MVP         | major vault protein | 1.99 1.82 | NM_001748.3     | CAPN2       | calpain 2 | 1.70 2.16 |
| NM_023938.4      | C1orf16     | chromosome 1 open reading frame 116 | 1.98 3.06 | NM_004344.1     | CETN2       | centrin | 1.70 1.54 |
| NM_024311.2      | MFSD11      | major facilitator superfamily domain containing 11 | 1.98 1.80 | NM_177947.2     | ARMXC3      | armadillo repeat containing, X-linked | 1.70 1.76 |
| NM_004433.3      | ELF3        | E74-like factor 3 | 1.97 3.72 | NM_000158.2     | GBE1        | glucan | 1.69 1.73 |
| NM_031229.2      | RBCK1       | RanBP-type and C3HC4-type zinc finger containing 1 | 1.97 4.21 | NM_052873.1     | C1orf179    | chromosome 14 open reading frame 179 | 1.69 1.52 |
| NM_005564.3      | LCN2        | lipocalin 2 | 1.97 4.19 | NM_023010.2     | MNT         | MAX binding protein | 1.69 1.51 |
| NM_002956.2      | CLIP1       | CAP-GLY domain containing linker protein 1 | 1.96 1.62 | NM_015367.2     | BCL2L13     | BCL2-like 13 | 1.69 1.88 |
| NM_005562.1      | LAMC2       | laminin, gamma 2 | 1.96 2.01 | NM_001005474.1  | NFKBIZ      | nuclear factor of kappa light polypeptide gene enhancer | 1.69 1.72 |
| NM_000332.2      | ATXN1       | ataxin 1 | 1.96 1.86 | NM_021202.1     | TPS3INP2    | tumor protein p53 inducible nuclear protein 2 | 1.69 1.89 |
| NM_001206.2      | KLF9        | Kruppel-like factor 9 | 1.96 2.19 | NM_138448.2     | ACYP2       | acylphosphatase 2 | 1.68 2.03 |
| NM_173359.3      | EIF4E       | eukaryotic translation initiation factor 4E family member 3 | 1.95 2.01 | NM_003729.1     | RTCD1       | RNA terminal phosphate cyclase domain 1 | 1.67 1.61 |
| NM_003565.1      | ULX1        | unc-51-like kinase 1 | 1.95 1.57 | NM_030912.2     | TRIM8       | tripartite motif-containing 8 | 1.67 1.70 |
| NM_012161.2      | FBXL5       | F-box and leucine-rich repeat protein 5 | 1.95 2.27 | NM_018202.3     | TMEM57      | transmembrane protein 57 | 1.67 1.88 |
| NM_001079864.1   | TAX1BP1     | Tax1 (human T-cell leukemia virus type I) binding protein 1 | 1.93 1.66 | NM_018297.2     | NGLY1       | N-glycanase 1 | 1.66 1.68 |
| NM_005063.1      | SCD         | stearyl-CoA desaturase | 1.93 4.44 | NM_183399.1     | RNF14       | ring finger protein 14 | 1.66 1.87 |
| NM_145279.4      | MOBKL2C     | MOB1, Mps One Binder kinase activator-like 2C | 1.92 2.58 | NM_032357.2     | CCDC15      | coiled-coil domain containing 15 | 1.66 1.63 |
| NM_004055.4      | CAPN5       | calpain 5 | 1.91 2.05 | NM_172037.2     | RDH10       | retinol dehydrogenase 10 | 1.66 2.20 |
| NM_173932.1      | PSMD13      | proteasome (prosome, macropain) 263 subunit | 1.91 2.14 | NM_012287.3     | CENTB2      | centaurin | 1.65 1.62 |
| ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change | ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change |
|------------------|-------------|------------|-------------|------------------|-------------|------------|-------------|
| NM_002885.1      | RAP1GAP     | RAP1 GTase activating protein | 1.91 | 2.02 | NM_018178.3 | GOLPH3L | golgi phosphoprotein 3-like | 1.65 | 1.64 |
| NM_145245.2      | EVL5        | ectopic viral integration site 5-like | 1.90 | 1.69 | NM_025147.3 | COQ10B | coenzyme Q10 homolog B | 1.64 | 1.54 |
| NM_021242.3      | MID1P1      | MID1 interacting protein 1 | 1.89 | 1.78 | NM_006285.2 | TESK1 | testis-specific kinase 1 | 1.64 | 1.61 |
| NM_006503.2      | PSMC4       | proteasome (prosome, macropain) 26S subunit | 1.89 | 2.16 | NM_001018102.1 | GRINL1A | glutamate receptor | 1.64 | 1.92 |
| NM_005975.2      | PTK6        | protein tyrosine kinase 6 | 1.88 | 1.97 | NM_005476.3 | GNE | glucosamine (UDP-N-acetyl)-2-epimerase | 1.64 | 2.98 |
| NM_207304.1      | MBNL2       | muscleblind-like 2 | 1.88 | 1.63 | NR_002204.1 | FTHL11 | ferritin, heavy polypeptide-like 11 on chromosome 8 | 1.63 | 1.79 |
| NM_000859.1      | HMGCR       | 3-hydroxy-3-methylglutaryl-Coenzyme A reductase | 1.88 | 2.46 | NM_053067.1 | UBQLN1 | ubiquilin 1 | 1.63 | 1.56 |
| NM_021021.2      | SNCTB1      | syntrophin | 1.88 | 1.64 | NM_005895.3 | GOLGA3 | golgi autoantigen | 1.63 | 1.58 |
| NM_032667.4      | BCL2        | Bernardinelli-Seip congenital lipodystrophy 2 | 1.87 | 1.73 | NM_037370.1 | CCNDBP1 | cyclin D-type binding-protein 1 | 1.63 | 1.50 |
| NM_006022.2      | TSC22D1     | TSC22 domain family | 1.87 | 2.40 | NM_001042430.1 | FAM164C | family with sequence similarity 164 | 1.62 | 2.01 |
| NM_012478.3      | WBP2        | WW domain binding protein 2 | 1.87 | 1.67 | NM_004751.1 | GCNT3 | glucosaminyl (N-acetyl) transferase 3 | 1.62 | 3.99 |
| NM_031476.2      | CRISPLD2    | cysteine-rich secretory protein LCLL domain containing 2 | 1.87 | 2.57 | NM_004428.2 | EFNA1 | ephrin-A1 | 1.61 | 2.10 |
| NM_017921.1      | NPLC4       | nuclear protein localization 4 homolog | 1.87 | 1.74 | NM_001030001.1 | RPS29 | ribosomal protein S29 | 1.57 | 1.61 |
| NM_015508.3      | TIPARP      | TCD2-inducible poly(ADP-ribose) polymerase | 1.87 | 1.75 | NM_018180.2 | DHX32 | DEAH (Asp-Glu-Ala-His) box polypeptide 32 | 1.61 | 1.52 |
| NM_001376.2      | DYNC1H1     | dynein, cytoplasmic 1, heavy chain 1 | 1.86 | 1.50 | NM_020202.2 | NIT2 | nitrilase family | 1.61 | 1.62 |
| NM_144693.1      | ZNF558      | zinc finger protein 558 | 1.86 | 1.98 | NM_032169.4 | ACAD11 | acyl-Coenzyme A dehydrogenase family | 1.61 | 1.73 |
| NM_003344.2      | UBE2H       | ubiquitin-conjugating enzyme E2H | 1.85 | 2.04 | NM_147223.2 | NCOA1 | nuclear receptor coactivator 1 | 1.60 | 1.57 |
| NM_152528.1      | WDHSUB1     | WD repeat, sterile alpha motif and U-box containing 1 | 1.85 | 1.74 | NM_001093771.1 | TXNRD1 | thioredoxin reductase 1 | 1.57 | 1.81 |
| NM_001225.3      | CAPS4       | caspase 4, apoptosis-related cysteine peptidase | 1.85 | 1.97 | NM_018317.1 | TBC1D19 | TBC1 domain family | 1.59 | 2.18 |
| NM_001512.2      | GSTA4       | glutathione S-transferase A4 | 1.84 | 2.45 | NM_002061.2 | GCLM | glutamate-cysteine ligase | 1.59 | 2.06 |
| NM_020299.3      | AKR1B10     | aldo-keto reductase family 1 | 1.83 | 2.42 | NM_019007.3 | ARMXC6 | armadillo repeat containing, X-linked 6 | 1.59 | 1.81 |
| NM_016143.3      | NSFL1C      | NSFL1 (p97) cofactor (p47) | 1.83 | 2.61 | NM_015974.2 | CRYL1 | crystallin, lambda 1 | 1.58 | 1.51 |
| NM_080655.1      | C0orf30     | chromosome 9 open reading frame 30 | 1.83 | 1.77 | NM_006149.2 | LGALS4 | lectin | 1.58 | 3.12 |
| NM_001018109.1   | PIR         | pinin | 1.83 | 2.67 | NM_012233.1 | RAB3GAP1 | RAB3 GTase activating protein subunit 1 | 1.57 | 2.78 |
| NR_002200.1      | FTHL2       | ferritin, heavy polypeptide-like 2 on chromosome 1 | 1.83 | 1.62 | NM_002808.3 | PSMD2 | proteasome (prosome, macropain) 26S subunit | 1.57 | 1.72 |
| NM_005485.3      | PARP3       | poly (ADP-ribose) polymerase family | 1.82 | 1.77 | NM_001030001.1 | MPS2 | ribosomal protein S29 | 1.57 | 1.61 |
| NM_058172.3      | ANTXR2      | anthrax toxin receptor 2 | 1.82 | 1.88 | NM_052849.2 | CCDC32 | coiled-coil domain containing 32 | 1.57 | 1.55 |
| NR_002166.1      | SECP1       | spondyloepiphysial dysplasia, late, pseudogene | 1.81 | 1.88 | NM_015484.4 | SYF2 | SYF2 homolog | 1.57 | 1.64 |
| NM_022449.1      | RAB17       | member RAS oncogene family | 1.80 | 1.64 | NM_001093771.1 | TXNRD1 | thioredoxin reductase 1 | 1.57 | 1.54 |
include the two cyclin-dependent kinase inhibitors, p21<sup>Cap1</sup> (CDKN1A) and p15<sup>ink4B</sup> (CDKN2B), which were up-regulated by 5.2- and 3.1- fold, 24 hr after GANT61 administration (Table 3). Additional genes required for the G1/S transition that were down-regulated include the E2 transcription factor E2F2 (-4.2-fold), and other critical genes that were down-regulated by 2.1- to 3.2- fold, include CYCLIN E (CCNE2), CDK2 and CDC25A. At G2/M, GANT61 induced down-regulated expression of CCNA2 (CYCLIN A2), CYCLIN B1 (CCNB1), CYCLIN B2 (CCNB2), CDK1 (CDC2), and CDC25C by 2.3- to 3.1- fold (Table 3).

To determine the robustness of cDNA microarray gene expression profiling following treatment of HT29 and GC3/c1 cells with GANT61 (20 µM) for 24 hr, qRT-PCR was employed to determine changes in expression of the selected group of 10 DEGs determined from the cDNA microarrays. The genes involved and primers synthesized are shown in Table 4. qRT-PCR was performed on cDNA generated using total RNA independently isolated from GANT61-treated HT29 and GC3/c1 cells for 0 hr, 16 hr, 24 hr, 38 hr and 48 hr after treatment. GAPDH was used to normalize all qRT-PCR data. Genes determined by qRT-PCR included the expression of E2F2, CCNE2, CDC25A and CDK2 at G1/S, which were down-regulated, up-regulation of CDKN1A and CDKN2B at G1/S, and down-regulation of CCNA2, CDC25C, CCNB2, and CDK1 at G2/M (Figure 5). The up-regulated or down-regulated changes

| ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change | ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change |
|------------------|-------------|------------|-------------|------------------|-------------|------------|-------------|
| NM_198310.2      | TTC8        | tetratricopeptide repeat domain 8 | 1.80 1.83   | NM_017707.2      | ASAP3       | ArfGAP with SH3 domain, ankyrin repeat and PH domain 3 | 1.57 1.59 |
| NM_002786.2      | PSMA1       | proteasome (prosome, macropain) subunit | 1.79 2.54   | NM_020412.3      | CHMP1B      | chromatin modifying protein 1B | 1.56 1.83 |
| NM_002796.2      | PSMB4       | proteasome (prosome, macropain) subunit | 1.78 1.53   | NM_017549.3      | EPDR1       | epednym related protein 1 | 1.56 1.52 |
| NR_002201.1      | FTHL3P      | fermentin | 1.78 2.10   | NM_001080493.2   | ZNF823      | zinc finger protein 823 | 1.56 1.56 |
| NM_020225.1      | STOX2       | storkhead box 2 | 1.78 5.98   | NM_001087838.2   | FNIP1       | folliculin interacting protein 1 | 1.56 1.50 |
| NM_080725.1      | SRXN1       | sulfedixin 1 homolog | 1.78 1.67   | NM_006343.2      | MERTK       | c-mr proto-oncogene tyrosine kinase | 1.56 2.12 |
| NM_015946.4      | PELO        | pelota homolog | 1.77 1.58   | NM_014851.2      | KHL2        | kelch-like 21 | 1.56 2.02 |
| NM_020751.1      | COG6        | component of oligomeric golgi complex 6 | 1.77 1.60   | NM_002860.3      | PSMC2       | proteasome | 1.55 1.77 |
| NM_001080538.1   | LOC441282   | similar to aldo-keto reductase family 1 | 1.77 2.31   | NM_145918.2      | CTSL1       | cathepsin L1 | 1.55 1.96 |
| NM_0004873.2     | GOLGB1      | golgin B1 | 1.76 1.82   | NM_003846.1      | PEX11B      | peroxisomal biogenesis factor 11 beta | 1.55 1.92 |
| NM_145040.2      | PRKCDBP     | protein kinase C | 1.76 3.27   | NM_014905.2      | GL5         | glutaminase | 1.55 2.21 |
| NM_201557.2      | FH2L2       | four and a half LIM domains 2 | 1.76 1.74   | NM_016154.3      | RAB8B       | member RAS oncogene family | 1.55 2.22 |
| NM_015037.2      | KIAA0913    | KIAA0913 | 1.75 1.52   | NM_052901.2      | SLC25A25    | solute carrier family 25 | 1.54 1.55 |
| NM_003003.2      | SEC14L1     | SEC14-like 1 | 1.74 1.94   | NM_033212.2      | CCDC102A    | coiled-coil domain containing 102A | 1.54 1.75 |
| NM_001031835.1   | PHKB        | phosphorylase kinase | 1.74 1.63   | NM_002799.2      | PSMB7       | proteasome (prosome, macropain) subunit | 1.54 1.56 |
| NM_016470.6      | C20orf111   | chromosome 20 open reading frame 111 | 1.74 1.54   | NM_001031716.1   | OBF2CA      | oligonucleotide/oligosaccharide-binding fold containing 2A | 1.53 1.60 |
| NM_001080791.1   | C15orf57    | chromosome 15 open reading frame 57 | 1.74 1.63   | NM_015187.3      | KIAA0746    | KIAA0746 protein | 1.53 1.62 |
| NM_002541.2      | OGDH        | oxoglutarate (alpha-ketoglutarate) dehydrogenase | 1.74 1.55   | NM_004034.1      | ANXA7       | annexin A7 | 1.52 1.75 |
| NM_015922.1      | NSDHL       | NAD(P) dependent steroid dehydrogenase-like | 1.74 2.30   | NM_002815.2      | PSMD11      | proteasome (prosome, macropain) 26S subunit | 1.52 1.71 |
| XM_001128220.1   | PLEKHM1     | pleckstrin homology domain containing, family M member 1 | 1.73 1.73   | NM_014889.2      | PITRM1      | pitrilysin metallopeptidase 1 | 1.52 2.15 |
| NM_004417.2      | DUSP1       | dual specificity phosphatase 1 | 1.73 1.78   | NM_007126.2      | VCP         | valosin-containing protein | 1.51 1.79 |
| NM_003971.3      | SPAG9       | sperm associated antigen 9 | 1.73 1.80   | NM_032017.1      | STK40       | serine/threonine kinase 40 | 1.50 1.66 |
| NM_016026.2      | RDH11       | retinol dehydrogenase 11 | 1.72 1.67   | NM_032776.1      | JMJDC1      | jumonji domain containing 1C | 1.50 1.81 |

doi:10.1371/journal.pone.0013054.t001

Table 1. Cont.
| ACCESSION NUMBER | GENE SYMBOL | DEFINITION                        | Fold Change | ACCESSION NUMBER | GENE SYMBOL | DEFINITION                        | Fold Change |
|------------------|-------------|-----------------------------------|-------------|------------------|-------------|-----------------------------------|-------------|
| NM_015086.1      | DDN         | dendrin                           | –6.81       | NM_014791.2      | MELK        | maternal embryonic leucine zipper kinase | –2.50 – 2.09 |
| NM_001013653.2   | LRRC26      | leucine rich repeat containing 26 | –4.77       | NM_016343.3      | CENPF       | centromere protein F, 350/400ka     | –2.50 – 2.71 |
| NM_004091.2      | E2F2        | E2F transcription factor 2        | –4.23       | NM_001789.2      | CDC25A      | cell division cycle 25 homolog A    | –2.50 – 2.45 |
| NM_182687.1      | PKMYT1      | protein kinase, membrane associated tyrosine/threonine 1 | –4.17      | NM_024037.1      | C1orf135    | chromosome 1 open reading frame 135 | –2.49 – 2.12 |
| NM_001013653.1   | LOC389816   | cytokeratin associated protein    | –3.85       | NM_017669.2      | ERCC6L      | excision repair cross-complementing rodent repair deficiency | –2.48 – 3.22 |
| NM_020675.3      | SPC25       | NDC80 kinetochore complex component, homolog | –3.51      | NM_031965.2      | GSG2        | germ cell associated 2 (haspin)     | –2.47 – 2.79 |
| NM_005733.1      | KIF20A      | kinesin family member 20A         | –3.45       | NM_018186.2      | C1orf112    | chromosome 1 open reading frame 112 | –2.47 – 1.88 |
| NM_002263.2      | KIFC1       | kinesin family member C1          | –3.36       | NM_001786.2      | CDC2        | cell division cycle 2, G1 to S and G2 to M | –2.47 – 2.63 |
| NM_006681.1      | NMU         | neuromedin U                      | –3.33       | NM_002105.2      | H2AFX       | H2A histone family, member X        | –2.46 – 2.86 |
| NM_014783.2      | ARHGAP11A   | Rho GTPase activating protein 11A | –3.33       | NM_018492.2      | PKB         | PDZ binding kinase                  | –2.46 – 2.21 |
| NM_006176.1      | NRGN        | neurogranin                       | –3.29       | NM_198516.1      | GALNT4      | UDP-N-acetyl-alpha-D-galactosamine  | –2.46 – 1.82 |
| NM_016095.1      | GINS2       | GINS complex subunit 2            | –3.25       | NM_018685.2      | ANLN        | anillin, actin binding protein      | –2.46 – 2.44 |
| NM_018063.3      | HELL5       | helicase, lymphoid-specific        | –3.24       | NM_002915.3      | RFC3        | replication factor C (activator 1) 3, 38kDa | –2.45 – 2.59 |
| NM_057735.1      | CCNE2       | cyclin E2                         | –3.22       | NM_007370.3      | RFC5        | replication factor C (activator 1) 5, 36.5kDa | –2.43 – 2.28 |
| NM_014750.3      | DLG7        | discs, large homolog 7             | –3.21       | NM_001032290.1   | PSRC1       | proline-serine-rich coiled-coil 1  | –2.43 – 2.53 |
| NM_001168.2      | BIRC5       | baculoviral IAP repeat-containing 5 | –3.18       | NM_000057.2      | BLM         | Bloom syndrome                     | –2.43 – 3.28 |
| NM_001255.2      | CDC20       | cell division cycle 20 homolog    | –3.12       | NM_012484.1      | HMMR        | hyaluronan-mediated motility receptor | –2.43 – 1.82 |
| NM_001237.2      | CCNA2       | cyclin A2                         | –3.11       | NM_145701.1      | CDC4A       | cell division cycle associated 4    | –2.43 – 2.26 |
| NM_144508.3      | CASC5       | cancer susceptibility candidate 5  | –3.09       | NM_024339.2      | THOC6       | THO complex 6 homolog              | –2.41 – 1.74 |
| NM_017611.2      | SLC43A3     | solute carrier family 43, member 3 | –3.05       | NM_003258.2      | TK1         | thymidine kinase 1                  | –2.41 – 3.74 |
| NM_018154.2      | ASF1B       | ASF1 anti-silencing function 1 homolog B | –3.03      | NM_182776.1      | MCM7        | minichromosome maintenance complex component 7 | –2.39 – 2.54 |
| NM_018410.3      | HJURP       | Holliday junction recognition protein | –3.02      | NM_178448.2      | C9orf140    | chromosome 9 open reading frame 140 | –2.39 – 2.34 |
| NM_004217.2      | AURKB       | aurora kinase B                    | –3.01       | NM_001067.2      | TOP2A       | topoisomerase (DNA) II alpha 170kDa | –2.39 – 3.16 |
| NM_002692.2      | POLE2       | polymerase (DNA directed), epsilon 2 (p59 subunit) | –3.01      | NM_178014.2      | TUB8        | tubulin, beta                       | –2.37 – 2.27 |
| NM_018101.2      | CDC4A       | cell division cycle associated 8   | –2.97       | NM_018193.2      | FANCI       | Fanconi anemia, complementation group I | –2.37 – 2.52 |
| NM_002875.2      | RAD51       | RAD51 homolog                      | –2.95       | NM_206833.2      | CTXN1       | cortexin 1                          | –2.36 – 2.04 |
| ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change | ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change |
|------------------|-------------|------------|-------------|------------------|-------------|------------|-------------|
| NM_003318.3      | TTK         | protein kinase | –2.94 – 2.82 | NM_031966.2      | CCNB1       | cyclin B1 | –2.36 – 2.28 |
| NM_001034.1      | RRM2        | ribonucleotide reductase | –2.91 – 2.80 | NM_005483.2      | CHAF1A      | chromatin assembly factor 1, subunit A (p150) | –2.36 – 2.21 |
| NM_004260.2      | RECQL4      | non-SMC condensin I | –2.90 – 3.66 | NM_005518.2      | HMGC52      | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 | –2.35 – 2.88 |
| NM_025049.2      | PIF1        | PIF1 5’-to-3’ DNA helicase homolog | –2.90 – 2.87 | NM_019013.1      | FAM64A      | family with sequence similarity 64 | –2.35 – 3.13 |
| NM_030928.2      | CDT1        | chromatin licensing and DNA replication factor 1 | –2.86 – 2.76 | NM_002129.2      | HMGB2       | high-mobility group box 2 | –2.35 – 2.18 |
| NM_016556.1      | PSMC3I      | PSMC3 interacting protein | –2.85 – 3.47 | NM_001034194.1   | EXOSC9      | exosome component 9 | –2.34 – 1.69 |
| NM_012177.2      | FBXO5       | F-box protein 5 | –2.78 – 2.95 | NM_001012413.1   | SGOL1       | shugoshin-like 1 | –2.33 – 2.85 |
| NM_018518.3      | MCM10       | minichromosome maintenance complex component 10 | –2.78 – 4.01 | NM_207418.2      | GCUD2       | gastric cancer up-regulated-2 | –2.32 – 2.28 |
| NM_001100118.1   | XRCC3       | X-ray repair complementing defective repair 3 | –2.69 – 2.49 | NM_006026.2      | NEK2        | NIMA (never in mitosis antigen) | –2.31 – 1.92 |
| NM_012415.2      | TPX2        | microtubule-associated, homolog | –2.72 – 3.22 | NM_024900.3      | PHF17       | PHD finger protein 17 | –2.28 – 1.67 |
| NM_054802.2      | TTK         | trophinin associated protein | –2.69 – 2.49 | NM_006026.2      | H1FX        | H1 histone family, member X | –2.24 – 2.08 |
| NM_001034194.1   | PSMC3I      | PSMC3 interacting protein | –2.68 – 2.81 | NM_003276.1      | TMPO        | thymopoietin | –2.24 – 1.95 |
| NM_014875.1      | KIF14       | kinesin family member 14 | –2.67 – 2.40 | NM_014875.1      | KIF14       | kinesin family member 14 | –2.23 – 2.12 |
| NM_001012413.1   | SGOL1       | shugoshin-like 1 | –2.66 – 2.08 | NM_014109.2      | ATAD2       | ATPase family, AAA domain containing 2 | –2.23 – 2.21 |
| ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change | ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change |
|------------------|-------------|------------|-------------|------------------|-------------|------------|-------------|
| NM_001254.3      | CDC6        | cell division cycle 6 homolog | -2.66 - 3.05 | NM_003390.2      | WEE1        | WEE1 homolog | -2.22 - 1.97 |
| NM_001034836.1  | RDM1        | RAD52 motif 1 | -2.65 - 2.65 | NM_002388.3      | MCM3        | minichromosome maintenance complex component 3 | -2.22 - 2.45 |
| NM_004526.2      | MCM2        | minichromosome maintenance complex component 2 | -2.65 - 3.43 | NM_005030.3      | PLK1        | polo-like kinase 1 | -2.22 - 2.37 |
| NM_004701.2      | CCNB2       | cyclin B2 | -2.65 - 2.52 | NM_005375.2      | MYB         | v-myb myeloblastosis viral oncogene homolog | -2.22 - 2.01 |
| NM_032818.2      | C9orf100    | chromosome 9 open reading frame 100 | -2.64 - 2.30 | NM_0024789.3     | TMEM180     | transmembrane protein 180 | -2.20 - 1.63 |
| NM_006397.2      | RNAEH2A     | ribonuclease H2, subunit A | -2.64 - 2.52 | NM_001012716.1   | C18orf56    | chromosome 18 open reading frame 56 | -2.20 - 2.30 |
| NM_014736.4      | KIAA0101    | KIAA0101 | -2.61 - 3.47 | NM_0017760.5     | NCAPG2      | non-SMC condensin II complex, subunit G2 | -2.20 - 2.11 |
| NM_004219.2      | PTTG1       | pituitary tumor transforming 1 | -2.61 - 2.23 | NM_005192.2      | CDKN3       | cyclin-dependent kinase inhibitor 3 | -2.20 - 1.98 |
| NM_182513.1      | SPC24       | SPC24, NDC80 kinetochore complex component, homolog | -2.61 - 3.15 | NM_006231.2      | POLE        | polymerase (DNA directed) | -2.19 - 3.53 |
| NM_198948.1      | NUDT1       | nudix (nucleoside diphosphate linked moiety X-type motif 1 | -2.59 - 3.09 | NM_018124.3      | RFWD3       | ring finger and WD repeat domain 3 | -2.19 - 1.56 |
| NM_033286.2      | C15orf23    | chromosome 15 open reading frame 23 | -2.58 - 1.77 | NM_006088.5      | TUBB2C      | tubulin, beta 2C | -2.19 - 1.63 |
| NM_024053.3      | CENPM       | centromere protein M | -2.57 - 3.45 | NM_198434.1      | AURKA       | aurora kinase A | -2.18 - 1.68 |
| NM_006479.3      | RAD51AP1    | RAD51 associated protein 1 | -2.57 - 2.54 | NM_007280.1      | OIP5        | Opa interacting protein 5 | -2.18 - 2.34 |
| NM_006739.3      | MCM5        | minichromosome maintenance complex component 5 | -2.56 - 3.21 | NM_014176.2      | UBE2T       | ubiquitin-conjugating enzyme E2T (putative). | -2.15 - 2.01 |
| NM_203401.1      | STMN1       | stathmin 1/oncoprotein 1B | -2.56 - 2.32 | NM_199420.3      | POLQ        | polymerase (DNA directed), theta | -2.14 - 2.45 |
| NM_013277.2      | RACGAP1     | Rac GTPase activating protein 1 | -2.55 - 1.79 | NM_001211.4      | BUB1B       | BUB1 budding uninhibited by benzimidazoles 1 homolog | -2.13 - 2.23 |
| NM_001005413.1   | ZWINT       | ZW10 interactor | -2.55 - 2.64 | NM_001025248.1   | DUT         | deoxyuridine triphosphatase | -2.13 - 2.36 |
| NM_004856.4      | KIF23       | kinesin family member 23 | -2.54 - 2.17 | NM_031217.2      | KIF18A      | kinesin family member 18A | -2.13 - 1.99 |
| NM_004523.2      | KIF11       | kinesin family member 11 | -2.53 - 2.12 | NM_0025108.2     | C16orf59    | chromosome 16 open reading frame 59 | -2.12 - 3.48 |
| NR_002734.1      | PTTG3       | pituitary tumor transforming 3 | -2.53 - 2.43 | NM_001042517.1   | DIAPH3      | diaphanos homolog 3 | -2.12 - 2.34 |
| NM_006027.3      | EXO1        | exonuclease 1 | -2.52 - 3.86 | NM_017998.1      | C9orf40     | chromosome 9 open reading frame 40 | -2.11 - 1.85 |
| NM_016448.1      | DTL         | denticencephal homolog | -2.51 - 3.12 | NM_0052844.3     | WDR34       | WD repeat domain 34 | -2.11 - 2.71 |
| NM_022770.2      | GINS3       | GINS complex subunit 3 (Psf3 homolog) | -2.10 - 1.65 | NM_017918.3      | CCDC109B    | coiled-coil domain containing 109B | -1.84 - 1.84 |
| NM_003503.2      | CDC7        | cell division cycle 7 homolog | -2.09 - 1.97 | NM_005782.2      | THOC4       | THO complex 4 | -1.84 - 1.63 |
| NM_001071.1      | TYMS        | thymidylate synthetase | -2.08 - 3.41 | NM_003035.2      | STIL        | SCL/TAL1 interrupting locus | -1.84 - 1.95 |
| NM_005441.2      | CHAF1B      | chromatin assembly factor 1, subunit B (p60) | -2.08 - 2.25 | NM_005342.2      | HMGB3       | high-mobility group box 3 | -1.83 - 1.60 |
| NM_014865.2      | NACPD2      | non-SMC condensin I complex, subunit D2 | -2.07 - 2.03 | NM_018132.3      | CENPQ       | centromere protein Q | -1.83 - 2.10 |
| ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change | ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change |
|------------------|-------------|------------|-------------|------------------|-------------|------------|-------------|
| NM_001798.2      | CDK2        | cyclin-dependent kinase 2 | −2.07 | NM_024516.2      | C16orf53    | chromosome 16 open reading frame 53 | −1.81 | |
| NM_002720.5      | DGC8        | DiGeorge syndrome critical region gene 8 | −2.07 | NM_015895.3      | GMNN        | geminin, DNA replication inhibitor | −1.81 | |
| NM_145018.2      | C11orf82    | chromosome 11 open reading frame 82 | −2.06 | NM_002689.2      | POLA2       | polymerase (DNA directed), alpha 2 (70kD subunit) | −1.81 | |
| NM_016195.2      | KIF20B      | kinesin family member 20B | −2.06 | NM_000154.1      | GALK1       | galactokinase 1 | −1.80 | |
| NM_001008393.1   | LOC201725   | hypothetical protein | −2.05 | NM_004499.3      | HNRNPAB     | heterogeneous nuclear ribonucleoprotein A/B | −1.80 | |
| NM_000465.1      | BARD1       | BRCA1 associated RING domain 1 | −2.05 | NM_024656.2      | GLT2D1      | glycosyltransferase 25 domain containing 1 | −1.80 | |
| NM_003173.2      | SUV39H1     | suppressor of variegation 3-9 homolog 1 | −2.05 | NM_199250.1      | C19orf48    | chromosome 19 open reading frame 48 | −1.79 | |
| NM_015426.2      | WDR51A      | WD repeat domain 51A | −2.05 | NM_153329.2      | ALDH16A1    | aldehyde dehydrogenase 16 family, member A1 | −1.79 | |
| NM_020342.1      | SLC39A10    | solute carrier family 39 | −2.05 | NM_032637.2      | SKP2        | S-phase kinase-associated protein 2 (p45) | −1.79 | |
| NM_144999.2      | LRR2C5      | leucine rich repeat containing 45 | −2.04 | NM_006191.2      | PA2G4       | proliferation-associated 2G4, 38kDa | −1.78 | |
| NM_000946.2      | PRIM1       | primase, DNA, polypeptide 1 (49kDa) | −2.04 | NM_001033580.1   | MYO19       | myosin XIX | −1.78 | |
| NM_016183.3      | MRTO4       | turnover 4 homolog | −2.04 | NM_017915.2      | C12orf48    | chromosome 12 open reading frame 48 | −1.78 | |
| NM_017518.5      | UCHL5P      | UCHL5 interacting protein | −2.04 | NM_003632.2      | UNG         | uracil-DNA glycosylase | −1.76 | |
| NM_020394.2      | ZNF695      | zinc finger protein 695 | −2.03 | NM_014985.2      | CEP152      | centrosomal protein 152kDa | −1.75 | |
| NM_203394.2      | EZF7        | EZF transcription factor 7 | −2.02 | NM_001333.2      | CTSL2       | cathepsin L2 | −1.75 | |
| NM_173529.3      | C18orf54    | chromosome 18 open reading frame 54 | −2.02 | NM_016310.2      | POLR3K      | polymerase (RNA) III (DNA directed) polypeptide K, 123 kDa | −1.75 | |
| NM_000156.4      | GAMT        | guanidinoacetate N-methyltransferase | −2.02 | NM_006406.1      | PRDX4       | peroxiredoxin 4 | −1.74 | |
| NM_007299.2      | BRCA1       | breast cancer 1, early onset | −2.02 | NM_005484.2      | PARP2       | poly (ADP-ribose) polymerase family, member 2 | −1.74 | |
| NM_014641.1      | MDC1        | mediator of DNA damage checkpoint 1 | −2.01 | NM_001039091.1   | PRPS2       | phosphoribosyl pyrophosphate synthetase 2 | −1.73 | |
| NM_032485.4      | MCM8        | minichromosome maintenance complex component 8 | −2.01 | NM_015703.3      | RRP7A       | ribosomal RNA processing 7 homolog A | −1.73 | |
| NM_024660.2      | TMEM149     | transmembrane protein 149 | −2.01 | NM_015032.1      | PDS5B       | PDS5, regulator of cohesion maintenance, homolog B | −1.73 | |
| NM_015414.2      | RPL36       | ribosomal protein L36 | −2.00 | NM_000992.2      | RPL29       | ribosomal protein L29 | −1.73 | |
| NM_021734.3      | SLC25A19    | solute carrier family 25, member 19 | −2.00 | NM_173608.1      | C14orf80    | chromosome 14 open reading frame 80 | −1.72 | |
| NM_003504.3      | CDC45L      | CDC45 cell division cycle 45-like | −2.00 | NM_001042550.1   | SMC2        | structural maintenance of chromosomes 2 | −1.72 | |
| NM_014708.3      | KNTC1       | kinetochore associated 1 | −1.99 | NM_014285.4      | EXOSC2      | exosome component 2 | −1.72 | |
| NM_018131.3      | CEP55       | centrosomal protein 55kDa | −1.98 | NM_138961.1      | ESAM        | endothelial cell adhesion molecule | −1.71 | |
| NM_007243.1      | NRM         | nurim | −1.98 | NM_145159.1      | JAG2        | jagged 2 | −1.70 | |
| NM_003517.2      | HIST2H2AC   | histone cluster 2, H2ac | −1.98 | NM_006392.2      | NOL5A       | nucleolar protein 5A (56kDa with KRE/D repeat) | −1.69 | |
| NM_001002800.1   | SMC4        | structural maintenance of chromosomes 4 | −1.98 | NM_033661.3      | WDR4        | WD repeat domain 4 | −1.69 |
| ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change | ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change |
|------------------|-------------|------------|-------------|------------------|-------------|------------|-------------|
|                 |             |            | HT29 GC3/c1 |                 |             |            | HT29 GC3/c1 |
| NM_001033505.1  | CSTF3       | cleavage stimulation factor, 3’- pre-RNA, subunit 3, 77kD | −1.97 −1.96 | NM_032358.2    | CCDC77     | coiled-coil domain containing 77 | −1.68 −1.54 |
| NM_181716.2     | PRR6        | proline rich 6 | −1.96 −1.85 | NM_018693.2    | FBXO11     | F-box protein 11 | −1.68 −1.93 |
| NM_018663.1     | PXMP2       | peroxisomal membrane protein 2, 23kDa | −1.96 −2.02 | NM_001026.3    | RPS24      | ribosomal protein 524 | −1.68 −1.94 |
| NM_005915.4     | MCM6        | minichromosome maintenance complex component 6 | −1.96 −2.25 | NM_016292.1    | TRAP1      | TNF receptor-associated protein 1 | −1.67 −1.51 |
| NM_005189.1     | CBX2        | chromobox homolog 2 | −1.96 −1.68 | NM_017613.2    | DONSON     | downstream neighbor of SON | −1.66 −1.61 |
| NM_001379.1     | DNMT1       | DNA (cytosine-5-) methyltransferase | −1.96 −1.84 | NM_006047.4    | RBM12      | RNA binding motif protein 12 | −1.66 −1.91 |
| NM_001002018.1  | HFC1R1      | host cell factor C1 regulator 1 (XPO1-dependent) | −1.96 −1.79 | NM_012140.3    | SLC25A10   | solute carrier family 25 (dicarboxylate transporter), member 10 | −1.66 −1.55 |
| NM_000234.1     | LIG1        | ligase I, DNA, ATP-dependent | −1.96 −2.81 | NM_001025238.1 | TSPAN4     | tetraspanin 4 | −1.66 −1.94 |
| NM_138443.2     | CCDC5       | coiled-coil domain containing 5 (spindle associated) | −1.95 −1.68 | NM_001008735.1 | HMG1L1     | high-mobility group (nonhistone chromosomal) protein 1-like 1 | −1.66 −1.62 |
| NM_020315.4     | PDXP        | pyridoxal (pyridoxine, vitamin B6) phosphatase | −1.95 −2.04 | NM_005956.2    | MTHFD1     | methylenetetrahydrofolate dehydrogenase (NADP-dependent) 1 | −1.65 −1.69 |
| NM_032117.2     | MND1        | meiotic nuclear divisions 1 homolog | −1.95 −2.63 | NM_145014.1    | HYL51      | hydroethalos syndrome 1 | −1.65 −1.62 |
| NM_194255.1     | SLC19A1     | solute carrier family 19 (folate transporter), member 1 | −1.95 −1.93 | NM_004309.3    | ARHGDA     | Rho GDP dissociation inhibitor (GDI) alpha | −1.64 −1.75 |
| NM_058216.1     | RAD51C      | RAD51 homolog C | −1.94 −2.13 | NM_182649.1    | PCNA       | proliferating cell nuclear antigen | −1.63 −1.99 |
| NM_018725.3     | IL17RB      | interleukin 17 receptor B | −1.94 −2.25 | NM_032118.2    | WDR54      | WD repeat domain 54 | −1.63 −1.77 |
| NM_024844.3     | NUP85       | nucleoporin 85kDa | −1.93 −1.53 | NM_015697.6    | COQ2       | coenzyme Q2 homolog | −1.63 −1.85 |
| NM_015190.3     | DNAJC9      | DnaJ (Hsp40) homolog, subfamily C, member 9 | −1.93 −1.64 | NM_032982.2    | CASP2      | caspase 2 | −1.63 −1.67 |
| NM_004237.2     | TRIP13      | thyroid hormone receptor-interactor 13 | −1.93 −2.23 | NM_001042762.1 | FIGN1L1    | fidgetin-like 1 | −1.63 −1.85 |
| NM_000179.1     | MSH6        | mutS homolog 6 | −1.93 −1.83 | NM_001100417.1 | UBR7       | ubiquitin protein ligase E3 component n-recognin 7 (putative) | −1.61 −1.64 |
| NM_001009936.1  | PHF19       | PHD finger protein 19 | −1.93 −2.42 | NM_003146.2    | SSR1P1     | structure specific recognition protein 1 | −1.60 −1.66 |
| NM_003609.2     | HIRIP3      | HIRA interacting protein 3 | −1.92 −2.01 | NM_152308.1    | C16orf75   | chromosome 16 open reading frame 75 | −1.60 −1.99 |
| NM_022145.3     | CENPK       | centromere protein K | −1.92 −2.25 | NM_001024662.1 | RPL6       | ribosomal protein L6 | −1.60 −1.56 |
| NM_022908.1     | NTSDC2      | 5’-nucleotidase domain containing 2 | −1.92 −1.81 | NM_182620.3    | FAM33A     | family with sequence similarity 33, member A | −1.59 −2.17 |
| NM_006342.1     | TACC3       | transforming acidic coiled-coil containing protein 3 | −1.92 −2.27 | NM_033120.2    | NKD2       | nacked cuticle homolog 2 | −1.59 −1.65 |
| NM_001080450.1  | KIAA1553    | KIAA1553 | −1.92 −1.65 | NM_003472.2    | DEK        | DEK oncogene (DNA binding) | −1.58 −1.91 |
| NM_001018115.1  | FANCD2      | Fanconi anemia, complementation group D2 | −1.92 −2.39 | NM_032015.3    | RNF26      | ring finger protein 26 | −1.58 −1.70 |
| NM_001033.2     | RRM1        | ribonucleotide reductase M1 polypeptide | −1.92 −1.87 | NM_018455.3    | CENPN      | centromere protein N | −1.57 −2.01 |
| NM_001078174.1  | SLC29A1     | solute carrier family 29 (nucleoside transporters) | −1.91 −2.19 | NM_003091.3    | SNRPB      | small nuclear ribonucleoprotein polypeptides B and B1 | −1.56 −1.52 |
in gene expression following GANT61 treatment and determined by cDNA microarray profiling, were confirmed by qRT-PCR (Figure 5).

**Discussion**

The HH signaling pathway is activated in a variety of human cancers following mutations in genes that regulate canonical HH signaling, including the receptor PTC, and the HH signaling molecule, SMO, and can also be activated via transcriptional up-regulation of the HH ligands (reviewed in [3]). This pathway is becoming of increasing importance due to gaining insight into its prominent role in many developmental processes, and in the maintenance of the malignant phenotype in a wide variety of human cancers, whose growth has been found to be prevented by selective inhibition of constitutive HH pathway activity [14,35,36]. Tumors of the brain, prostate, skin, pancreas, and kidney have demonstrated the requirement for HH-GLI signaling, and have responded to inhibition of the HH signaling target molecule SMO by cyclopamine or SMOshRNA [4,19,20,21,22,23].

The transcriptional activators in HH signaling comprise members of the GLI family of transcription factors, GLI1 and GLI2, which have both distinct as well as overlapping functions [16]. Activation of the GLI proteins is an intricate process that involves modifications and interactions of a number of positive and negative pathway regulators and is not fully understood [1,14,35]. Target genes regulated by the HH signaling pathway differ between tissues and cell types, as well as being influenced by the presence or absence of regulatory factors co-expressed with GLI pathways. Thus, oncogenic signaling pathways converge on canonical HH signaling at the level of the GLI transcription factors and additionally on target genes downstream of GLI1 and GLI2 to further drive the HH signaling pathway in cellular survival in malignancies [6,7,20,38,39,40]. The HH signaling phenotype is therefore significantly influenced by the presence or absence of regulatory factors co-expressed with GLI and ultimately determined by the co-expression of additional regulatory factors, and hence by the cellular context of gene expression.

HH signaling plays a role in the differentiation program of normal intestinal villi [11,12,41], and it has been suggested recently that human colon cancer epithelial cells display a HH-GLI signaling axis in the process of carcinogenesis [24,25]. Expression of HH-GLI pathway components was consistently

| ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change | ACCESSION NUMBER | GENE SYMBOL | DEFINITION | Fold Change |
|------------------|-------------|------------|-------------|------------------|-------------|------------|-------------|
| NM_153485.1      | NUP155      | nucleoporin 155kDa | –1.91 – 1.65 | NM_003863.2      | DPM2        | dolichyl-phosphate mannosyltransferase polypeptide 2 | –1.56 – 1.77 |
| NM_002482.2      | NASP        | nuclear autoantigenic sperm protein (histone-binding) | –1.91 – 1.96 | NM_017916.1      | PIH1D1      | PIH1 domain containing 1 | –1.55 – 1.97 |
| NM_176880.4      | NR2C2AP     | nuclear receptor 2C2-associated protein | –1.90 – 1.87 | NM_020810.1      | TRMT5       | tRNA methyltransferase S homolog | –1.55 – 1.54 |
| NM_021953.2      | FOXM1       | forkhead box M1 | –1.90 – 2.28 | NM_058219.2      | EXOSC6      | exosome component 6 | –1.55 – 1.65 |
| NM_015721.2      | GEMIN4      | gem (nuclear organelle) associated protein 4 | –1.90 – 1.54 | NM_002882.2      | RANBP1      | RAN binding protein 1 | –1.54 – 1.88 |
| NM_018188.2      | ATAD3A      | ATPase family, AAA domain containing 3A | –1.89 – 1.70 | NM_007111.3      | TFDP1       | transcription factor Dp-1 | –1.54 – 1.51 |
| NM_145060.3      | C18orf24    | chromosome 18 open reading frame 24 | –1.89 – 2.45 | NM_021709.1      | SIVA        | CD27-binding (Siva) protein | –1.54 – 1.71 |
| NM_018365.1      | MNS1        | meiosis-specific nuclear structural 1 | –1.89 – 2.12 | NM_015140.2      | TLL12       | tubulin tyrosine ligase-like family, member 12 | –1.53 – 1.61 |
| NM_012310.3      | KIF4A       | kinesin family member 4A | –1.89 – 2.38 | NM_022044.2      | SDF2L1      | stromal cell-derived factor 2-like 1 | –1.53 – 1.70 |
| NM_015261.2      | NCPD3       | non-SMC condensin II complex, subunit D3 | –1.88 – 1.88 | NM_002346.1      | LY6E        | lymphocyte antigen 6 complex, locus E | –1.52 – 1.56 |
| NM_013296.3      | GPSM2       | G-protein signalling modulator 2 | –1.87 – 1.63 | NM_022490.1      | POLR1E      | polymerase (RNA I) polypeptide E, 53kDa | –1.52 – 1.77 |
| NM_002691.1      | POLD1       | polymerase (DNA directed), delta 1, catalytic subunit | –1.87 – 2.22 | NM_032799.4      | ZDHHC12     | zinc finger, DHHC-type containing 12 | –1.51 – 1.64 |
| NM_203467.1      | PPLS        | peptidylprolyl isomerase (cyclophilin)-like 5 | –1.87 – 1.79 | NM_001017980.2   | LOC203547   | hypothetical protein | –1.51 – 1.54 |
| NM_015201.3      | BOP1        | block of proliferation 1 | –1.87 – 2.29 | NM_003801.3      | GPAA1       | glycosylphosphatidylinositol anchor attachment protein 1 homolog | –1.51 – 1.89 |
| NM_018353.3      | C14orf106   | chromosome 14 open reading frame 106 | –1.86 – 1.54 | NM_016594.1      | FKBP11      | FK506 binding protein 11, 19 kDa | –1.50 – 1.94 |
| NM_080626.5      | BR3BP       | BR3 binding protein | –1.84 – 1.72 | NM_002754.4      | SFXN1       | siderolflexin 1 | –1.50 – 1.88 |

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Table 2. Cont.
HH-Dependent Gene Expression

Figure 3. The top 15 canonical signaling pathways influenced by inhibition of GLI1/GLI2 function in HT29 and GC3/c1 cells. The top 15 canonical signaling pathways, determined by IPA, that were significantly up-regulated or down-regulated by GANT61 treatment in HT29 and GC3/c1 cells, are shown. The 1,368 DEGs in HT29 and 1,002 DEGs in GC3/c1 were mapped to the IPA-defined network. The significance p-values that determine the probability that the association between the genes in the dataset and the canonical pathway is by chance alone were calculated by Fisher’s exact test, and are expressed as −log(p-value). A. Pathways with enriched down-regulated genes. B. Pathways with enriched up-regulated genes. Blue: Pathways common to both HT29 and GC3/c1. Red: Pathways unique to either HT29 or GC3/c1. Yellow squares: Ratio of the number of DEGs that map to a specific canonical pathway divided by total number of genes that make up that pathway.

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demonstrated in an analysis of 40 primary human colon carcinomas and tumors metastatic to the liver [26], consistent with findings of previous investigators [23,42,43]. Thus, using qRT-PCR, the expression of GLI1, PTCH1, GLI2 and SHH was determined in all human colon carcinomas examined. The requirement for both GLI1 and GLI2 for sustained proliferation and survival of human colon carcinoma cell lines in vitro, including HT29, was demonstrated using siRNA technology [26]. In addition, knockdown of SMO by SMOshRNA prevented the growth of HT29 cells in SCID mice, while wt HT29 subcutaneous xenografts responded to cycloamine by reduction in tumor volume [26]. Thus, canonical activation of GLI1 and GLI2 via SMO is important for the survival and proliferation of human colon carcinoma cells in vivo.

In the current study, the function of both GLI1 and GLI2 downstream of SMO was inhibited in the presence of GANT61, a small molecule inhibitor that was identified from a cell-based screen to specifically inhibit GLI1-mediated transcription, but that also inhibited the function of GLI2 [34]. This agent was selected to specifically inhibit the final arbiters of HH signaling, the GLI transcription factors, in elucidation of the downstream target genes that determine HH-dependent proliferation in human colon carcinoma cells. Two cell lines, well characterized in our laboratories, HT29 and GC3/c1, were treated with GANT61 (20 μM) for 24 hr, and the expression of GLI1, GLI2 and PTCH1 mRNA was down-regulated. Further, the effects on cellular proliferation as determined by the distribution of cells within the cell cycle and flow cytometric analysis demonstrated accumulation of cells in G1 following treatment, with a concomitant decrease of cells from the G2/M compartment, and in the case of HT29, also from S-phase, suggesting the induction of a G1/S checkpoint.

HT29 and GC3/c1 cells were subsequently treated with GANT61 (20 μM) for 24 hr, RNA was extracted, and changes in gene expression were determined by Illumina cDNA microarray profiling. Following statistical analyses, 1,368 genes in HT29 and 1,002 genes in GC3/c1, were determined to be significantly modulated by GANT61 treatment (FC > 1.5; p < 0.001). For genes that were up-regulated in expression, 296 genes were common to both cell lines, and for down-regulated genes, 309 genes were common to both cell lines. The blockade of cells at the G1/S boundary is evidenced by up-regulated expression of p21^Cip1 and p15^Inkb4, that in part regulate the G1/S transition. p15^Inkb4 is a member of the Ink4 family of CDK inhibitors, is induced in response to cytostatic signals [32,44], and complexes with CYCLIN D/CDK4 or CYCLIN D/CDK6 to mediate G1-phase arrest at the G1/S transition in certain systems [30,32]. p21^Cip1 can bind a broad range of cyclin-CDK complexes, with a preference for those containing CDK2 (reviewed in [31,43], and during a normal cell cycle, facilitates active cyclin-CDK complex formation to promote cell proliferation. However when overexpressed, p21^Cip1 forms an inhibitory complex with CYCLIN E/CDK2, leading to G1- and consequently S-phase arrest, thereby forming the G1/S checkpoint [46,47]. The scheduled timing of expression of CYCLINS E, A and B that drive cell cycle progression, is reflected in the major changes in the phases of the cell cycle. CYCLIN E is expressed at maximal levels in cells undergoing the G1 to S transition, declining during S-phase progression, such that G2/M cells are CYCLIN E-negative (reviewed in [30]). CYCLIN A is expressed in late G1, demonstrates pronounced expression during S-phase, and increases as the cells advance towards G2, with degradation in early mitosis; B type cyclins begin to be expressed in late S-phase, and drive the cells though G2- and M-phases of the cell cycle [30]. CDK2 controls the G1/S transition by complexing with CYCLIN E, and is activated by CDC25A, which dephosphorylates CDK2 [48]. CDK2 controls cellular entry into mitosis at the G2/M transition, thereby forming complexes with CYCLINS A and B, is activated by CDC25C, and is down-regulated in late M-phase [29]. All of these genes are down-regulated in expression in response to the inhibition of GLI1/GLII2 function (Table 3). Thus, signals elicited to promote cellular accumulation at G1/S lead to the repression of genes that regulate further cell cycle progression, and p21^Cip1 expression has been shown to deplete the expression of genes that regulate DNA replication and repair, and mitosis [49,50,51]. In the current study these genes include CDC6 (active at the G1/S transition and essential for the initiation of DNA replication), TYMS, TOP2A, TK1, POLE and POLE2 (S-phase), and AURKB and CDC20 (mitosis [52,53]), determined by heat map analysis. A schematic representation of the genes involved in GANT61-induced inhibition of cell cycle progression at G1/S, S-phase progression, and regulation during G2- and M-phases, identified from cDNA microarrays, heat map analysis, and by qRT-PCR, is shown in Figure 6, and involves 5 of the 12 common signaling pathways determined by IPA analysis.

Comprehensive cDNA microarray gene profiling analysis of genes that determine the HH signaling phenotype has been conducted only in non-cancer cell models. In these systems, GLI activation has been stimulated by EGF treatment [6], stable GLI1 or HA-RAS expression [7], or expression of constitutively activated GLI2 [16]. In these studies, CYCLIN D [6,7], GADD153 [7], CDKN2B, CDKN1A, CDK2, PCNA, TOP2A, CCNB1, XRCC1 [16], have been identified as genes activated downstream of GLI. In GANT61-treated human colon carcinoma cells, novel DNA damage-inducible transcripts DDIT3 (GADD153), DDIT4 (REDD1), DDIT2 (GADD45G), PPAR-γ15A (GADD34) and ATF3 were significantly up-regulated concomitant with the arrest of cells at G1/S. TP53INP1, involved in cell cycle regulation, and TP53INP2, linked to cell death responses, were also up-regulated. Additional novel genes involved in S-phase progression and DNA damage response that were significantly down-regulated include KIAA0101 (p15[PAF]), Replication Factor C variants 2, 3, 4, 5, CDT1, the E2F transcription factors CDC24 and TFDPI, MDC1, PCNA, FANC D2, and the genes involved in DNA repair, RAD51C (XRCC3), RAD54B, RAD51 and HELL5.

In summary, we have compared gene expression profiles in two human colon carcinoma cell lines after targeting the function of the transcriptional regulators of HH signaling, GLI1 and GLI2, using the small molecule inhibitor GANT61. Data are consistent with accumulation of cells at the G1/S boundary, as evidenced from flow cytometric analysis, cDNA microarray gene profiling, and qRT-PCR. GANT61-treated cells demonstrated up-regulat-
Figure 4. Heat map showing fold change patterns of most highly DEGs in GANT61-treated human colon carcinoma cell lines. The heat map was generated in Matlab (Mathworks), and compares fold change patterns of the most highly DEGs in HT29 and GC3/c1 cells after GANT61 treatment. The most highly DEGs demonstrated a differential expression p-value of $p < 0.001$ between vehicle control (0.2% DMSO) and GANT61-treated cells. Left panel (red): up-regulated genes. Right panel (green): down-regulated genes. Genes denoted with asterisks define those genes with
ed expression of the CDK inhibitors p21\(^{Cip1}\) and p15\(^{Ink4b}\), that function at the G1/S boundary, and down-regulated expression of additional key genes that determine the G1/S transition, initiation of DNA replication, S-phase progression, DNA repair, and subsequent transition through the G2/M phases. Inhibition of the transcriptional regulation of HH signaling in human colon carcinoma cells therefore directly involves genes that regulate cell cycle transition through G1/S, cell cycle progression, and proliferation, and genes involved in stress-induced and DNA damage responses.

**Materials and Methods**

**Human colon carcinoma cell lines**

HT29 was purchased from ATCC (Manassas, VA), while GC3/c1 was established in culture by our group from a human colon adenocarcinoma xenograft model [54]; both cell lines express mutant p53 alleles. Cell lines were maintained in the presence of folate-free RPMI 1640 medium containing 10% dFBS and 80 nM [6RS] 5-methyltetrahydrofolate.

**Flow cytometric analysis**

HT29 and GC3/c1 cells were plated at a density of 100,000 cells/well in six-well plates. After overnight attachment, cells were treated with GANT61 (20 μM; Enzo Life Sciences, Germany) or vehicle control (DMSO, 0.2%), in duplicate, for 24 hr, followed by washing \(\times 1\) with PBS, trypsinization, and centrifugation. Cells were fixed with 70% ethanol at RT, 20–30 min, stored at \(-20^\circ\)C overnight, centrifuged for 5 min at 200 \(\times g\) to remove ethanol, and washed \(\times 1\) in PBS. The cells were resuspended in PBS, and low molecular weight DNA was extracted using DNA extraction buffer (0.2M \(\text{Na}_2\text{HPO}_4\) and 0.1M citric acid, pH 7.8) for 5 min. The extracted DNA was centrifuged and resuspended in DNA staining solution containing propidium iodide (50 μg) and DNAse-free RNase (2 mg), and allowed to incubate for 30 min in the dark at RT. Distribution of cells throughout the cell cycle was analyzed using a FACSCalibur flow cytometer, and data were analyzed using CellQuest software.

**RNA isolation**

Cells were seeded at \(7 \times 10^6\) cells/10 cm plate overnight at \(60\%\) confluency, and subsequently treated with either vehicle control (0.2% DMSO) or GANT61 (20 μM), in duplicate, for 24 hr. Cells were subsequently harvested and RNA was isolated using the RNeasy® Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. Integrity of the RNA was determined by spectrophotometry and electrophoresis.

**cDNA microarray analysis**

RNA (250 ng) was reverse transcribed into cRNA and biotin-UTP labeled using the Illumina® TotalPrep RNA Amplification Kit (Ambion, Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. cRNA was quantified using a nanodrop spectrophotometer, and the cRNA quality (size distribution) further analyzed on a 1% agarose gel. Biotinylated cRNAs were hybridized to the Illumina Human-ref8 V3.0 BeadChip (Illumina, San Diego, CA) that represents 18,401 genes, using standard protocols. The arrays were washed and subsequently scanned using an Illumina BeadArray Reader.

Raw signal intensities of gene expression data were processed and analyzed using GenomeStudio (Illumina), with background subtraction and average normalization performed on the average signal intensities, and p-values were calculated. Raw data were exported from GenomeStudio into Excel (available as Data S1 and Data S2), and the fold change in gene expression calculated, by dividing the average gene expression signal intensity of treated samples (GANT61) with that of the vehicle control (DMSO). Differential gene expression (DEG) analysis between control and GANT61-treated samples, was subsequently conducted using the Illumina customer model, which applies multiple testing corrections that determines the false discovery rate (FDR; [55]). Thus, FDR-adjusted differential scores and p-values for each gene/probe set between treated and control samples were generated. Genes with a FDR-adjusted p-value of \(<0.001\) and fold change \(\geq 1.5\) were considered to be DEGs and were subjected to Venn analysis and Ingenuity Pathway analysis.

**Table 3.** Gene expression changes from cDNA arrays at G1/S and G2/M.

| Cell Cycle Phase | Accession Number | Gene Symbol | Gene Name | Fold Change |
|-----------------|-----------------|-------------|-----------|-------------|
|                 |                 |             |           | HT29        | GC3/c1      |
| G1/S            | NM_000389.2     | CDKN1A      | cyclin-dependent kinase inhibitor 1A (p21\(^{Cip1}\)) | +5.21        | +2.02       |
|                 | NM_007487.2     | CDKN2B      | cyclin-dependent kinase inhibitor 2B (p15\(^{Ink4b}\)) | +3.13        | +2.13       |
|                 | NM_004091.2     | E2F2        | E2F transcription factor 2 | -4.24        | -2.26       |
|                 | NM_057735.1     | CCNE2       | cyclin E2 | -3.22        | -3.24       |
|                 | NM_001789.2     | CDC25A      | cell division cycle 25 homolog A | -2.50        | -2.45       |
|                 | NM_001798.2     | CDK2        | cyclin-dependent kinase 2 | -2.07        | -1.88       |
| G2/M            | NM_000389.2     | CCNA2       | cyclin A2 | -3.11        | -3.01       |
|                 | NM_004701.2     | CCNB2       | cyclin B2 | -2.65        | -2.52       |
|                 | NM_001786.2     | C DK1       | cyclin-dependent kinase 1 | -2.47        | -2.63       |
|                 | NM_031966.2     | CCNB1       | cyclin B1 | -2.36        | -2.28       |
|                 | NM_028809.2     | CDC25C      | cell division cycle 25 homolog C | -2.27        | -2.05       |

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These differentially expressed genes were uploaded and mapped to the library of canonical pathways of the Ingenuity Pathways Analysis (IPA) database for pathway analysis (Ingenuity Systems, http://www.ingenuity.com, Mountain View, CA). The mapped datasets containing either up-regulated or down-regulated genes in HT29 or GC3/c1 cells with corresponding expression values were subjected to core analysis that includes overall canonical pathway enrichment analysis. The significance of enrichment of genes mapped to different canonical pathways was calculated by the Fischer’s exact test (p-value) to determine the probability that the association between the genes and the canonical pathway could be explained by chance alone. Further, the ratio between the number of identified genes in a particular pathway and total number of genes that make up that pathway provides an estimation of the extent of pathway involvement. The pathway enrichment analysis was performed to ascertain that only the expected products had been amplified back to 95°C with 40 cycles of primer annealing and extension at 60°C.

Fold change patterns of most highly DEGs in HT29 and GC3/c1 were compared by Heat map analysis using Matlab software. DEGs analyzed and displayed in the heat map demonstrated a differential expression p-value of p<0.001 between control and GANT61 treated cells in both cell lines.

### Quantitative Real-Time PCR (qRT-PCR)

The expression levels of selected genes identified by cDNA microarray expression profiling at 24 hr following GANT61 treatment, were validated by qRT-PCR. Thus, HT29 or GC3/c1 cells were either untreated (vehicle control, 0.2% DMSO), or treated with GANT61 (20 μM) for 0 hr, 16 hr, 24 hr, 38 hr or 48 hr at 37°C, dissolved in DMSO-containing medium. Total RNA (1 μg) was employed to prepare cDNA via reverse transcription using the iScript Select cDNA Synthesis Kit (Biorad) Reverse Transcription System according to manufacturers instructions and analyzed using an Applied Biosystems 7500 PCR Detection System (Applied Biosystems Inc.). All amplifications were primed by pairs of chemically synthesized 18- to 24- mer oligonucleotides designed using freely available primer design software (Primer-BLAST, NCBI) to generate target amplicons of 50–547 bp. All reactions were performed in a final volume of 15 μl. qRT-PCR reaction conditions were as follows: activation at 95°C for 10 min with 40 cycles of denaturation at 95°C for 15 s, primer annealing and extension at 60°C for 1 min and ramping back to 95°C. Melt curve analysis of all samples was routinely performed to ascertain that only the expected products had been generated. A fluorescence reading determined the extent of amplification at the end of each cycle. mRNA expression levels of target genes were normalized to the expression of glyceraldehyde-phosphate dehydrogenase (GAPDH) and quantified using the comparative CT method [56]. Q-RT-PCR for each gene was analyzed in selected pathways pertaining to cell cycle progression containing both up-regulated and down-regulated genes were also shown in a histogram of pathway vs. –log (p-value). Datasets enriched canonical pathways were ranked by the ratio between the number of identified genes in a particular canonical pathway could be explained by chance alone. Further, the probability that the association between the genes and the canonical pathway could be explained by chance alone. Further, the ratio between the number of identified genes in a particular pathway and total number of genes that make up that pathway provides an estimation of the extent of pathway involvement. The pathway enrichment analysis was performed to ascertain that only the expected products had been amplified back to 95°C with 40 cycles of primer annealing and extension at 60°C.

### Table 4. Sequences of primers used in quantitative Real-Time PCR.

| Accession # | Gene Symbol | Strand | Primer Sequence | Product Size (bp) |
|-------------|-------------|--------|-----------------|-------------------|
| NM_078487.2 | CDKN2B      | Plus   | 5’-TCTCGGTGGCGGGGAGTCA-3’ | 95 |
|             |             |        | 5’-TGGCCAGCTTGCGACGGTTG-3’ | |
| NM_004701.2 | CCNB2       | Plus   | 5’-CTAACGGGCCTGATGCGGCT-3’ | 54 |
|             |             |        | 5’-CAGGGAGGACCCCGGCTGA-3’ | |
| NM_057749.1 | CCNE2       | Plus   | 5’-GGGCCAGTGGTGGGCGG-3’ | 94 |
|             |             |        | 5’-GGGCTGCTGCTGCTG-3’ | |
| NM_001789.2 | CDC25A      | Plus   | 5’-CTGTTGTTGCGACTCTCGC-3’ | 159 |
|             |             |        | 5’-GGTCGTCAAGGTTGACCTGG-3’ | |
| NM_001798.3 | CDK2        | Plus   | 5’-TTTGGCTGAATGTTGACCTCGGG-3’ | 159 |
|             |             |        | 5’-CCGCGCCCACTTGGGAAAC-3’ | |
| NM_004091.2 | E2F2        | Plus   | 5’-CCGCGACACGTGGTGTA-3’ | 97 |
|             |             |        | 5’-GGCGTCTGCTGCTGCACTGG-3’ | |
| NM_001237.3 | CCNA2       | Plus   | 5’-AAAGGCAGCCGCGCTCAA-3’ | 89 |
|             |             |        | 5’-CTGTTGGCTGCTGCTGAGCCC-3’ | |
| NM_005631.3 | CDKN1A      | Plus   | 5’-CTGCCGCCAGCGTGATGTA-3’ | 189 |
|             |             |        | 5’-GCTGCTGCTGCTGCCACTGG-3’ | |
| NM_022809.2 | CDC25C      | Plus   | 5’-TGTCATTTAGCTGCTGGTA-3’ | 189 |
|             |             |        | 5’-GGGCCAGTCTGCTGTA-3’ | |
| NM_001786.2 | CDK1        | Plus   | 5’-ACTGCGTATTGCGCCGCGCC-3’ | 118 |
|             |             |        | 5’-TGAGTAAGCAGCTGCTGAGCCAACCA-3’ | |
| NM_005269   | GLI1        | Plus   | 5’-GCCGCAGACAGGAGGCCACTC-3’ | 547 |
|             |             |        | 5’-CTCGACGCTCAAGGCGACCA-3’ | |
| NM_005270   | GLI2        | Plus   | 5’-CAGCGGTGCTGCTGCTGAGGA-3’ | 227 |
|             |             |        | 5’-CTCGACGCTCAAGGCGACCA-3’ | |
| NM_000264   | PTCH1       | Plus   | 5’-CCACAGAAGGCCCTCTACA-3’ | 214 |
|             |             |        | 5’-CTGTAATTTGGCGCCCTCC-3’ | |

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Figure 5. Selected DEGs from cDNA array gene expression profiling analyzed by qRT-PCR in HT29 (A) or GC3/c1 (B). Cells were treated with vehicle alone (0.2% DMSO) or GANT61 (20 μM) for 16 hr, 24 hr, 38 hr, or 48 hr. Total RNA was extracted and qRT-PCR was performed as described in Materials and Methods using the primer sets listed in Table 2. Data represent the mean±SD of 4 determinations, and GAPDH was used to normalize the relative mRNA levels.

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Figure 6. Schematic representation of genes involved in GANT61-induced inhibition of cell cycle progression. From cDNA microarray, heat map, and qRT-PCR analyses, genes involved at different phases of the cell cycle including the G1/S transition, and progression through S-, G2-, and M- phases, are shown. The genes identified include CDK inhibitors, members of the CDK and CDC families, cyclins, genes involved in DNA replication and repair, and genes that regulate the mitotic spindle, and involve 5 of the 12 common signaling pathways determined by IPA analysis. Red: Up-regulated genes. Green: Down-regulated genes. Light shade—dark shade, increasing differential expression.

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determined in duplicate, and each experiment was repeated at least twice.

Supporting Information

Data S1 HT29 Raw Microarray Data. Found at: doi:10.1371/journal.pone.0013054.s001 (11.06 MB XLS).

Data S2 GC3/c1 Raw Microarray Data. Found at: doi:10.1371/journal.pone.0013054.s002 (11.06 MB XLS).

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Author Contributions

Conceived and designed the experiments: TM JAH. Performed the experiments: TS MJ AA. Analyzed the data: TS ZD. Contributed reagents/materials/analysis tools: ZHD. Wrote the paper: TS JAH. Directed the whole project from conceptual design to experimental execution, and preparation of the text of the manuscript: JAH. Data interpretation: TM JD AA. Data presentation design: ZHD AA.
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