Gene Expression Profiling Leads to Identification of GLI1-binding Elements in Target Genes and a Role for Multiple Downstream Pathways in GLI1-induced Cell Transformation*

The zinc finger transcription factor GLI1, which mediates Sonic hedgehog signaling during development, is expressed in several human cancers, including basal cell carcinoma, medulloblastoma, and sarcomas. We identified 147 genes whose levels of expression were significantly altered in RNA obtained from cells demonstrating a transformed phenotype with stable GLI1 expression or stable Ha-ras expression. Comparison of expression profiles from GLI1- and Ha-ras-expressing cells established a set of genes unique to GLI1-induced cell transformation. Thirty genes were altered by stable GLI1 expression, and 124 genes were changed by stable Ha-ras expression. Seven genes had altered expression levels in both GLI1- and Ha-ras-expressing cells. Genes whose expression was altered by GLI1 included cell cycle genes, cell adhesion genes, signal transduction genes, and genes regulating apoptosis. GLI1 consensus DNA-binding sequences were identified in the 5′ regions of cyclin D2, IGFBP-6, osteopontin, and plakoglobin, suggesting that these genes represent immediate downstream targets. Gel shift analysis confirmed the ability of the GLI1 protein to bind these sequences. Up-regulation of cyclin D2 and down-regulation of plakoglobin were demonstrated in GLI1-amplified compared with non-amplified human rhabdomyosarcoma cells. Many of the GLI1 targets with known function identified in this study increase cell proliferation, indicating that GLI1-induced cell transformation occurs through multiple downstream pathways.

Important gene hierarchies, in part coding for components of signal transduction pathways, regulate growth and differentiation during development. One such pathway is the Sonic hedgehog-Patched-Gli pathway (1). SHH1 signaling is critical to the genetic specification of fate of many tissues during early organogenesis including the central nervous system (2, 3), lung (4), prostate (5), bone (6–8), and muscle (9). SHH signaling is mediated by the GLI family of transcription factors (10). One of these genes, GLI1, has been shown to be a transcriptional activator operating through a C-terminal VP-16-like acidic helical domain (11). GLI1 transforms cells in culture, and its expression is associated with significant human cancers including basal cell carcinoma (12), medulloblastoma (13), and sarcomas (14). Few downstream targets of GLI1 are known, which precludes a clear understanding of its action in carcinogenesis. Genetic evidence suggests that PTC and Wnt genes are downstream targets of GLI1 (15), and biochemical evidence has established HNF-3β (Hepatocyte Nuclear Factor-3β) as a target of GLI1 during development (16).

Microarray technology has provided a methodology to study the expression of thousands of genes simultaneously and has been used in many important settings (17). Among these is the dissection of signal transduction pathways. To identify unique downstream targets of GLI1, we have utilized a cell transformation phenotype as a selection system for the stable integration and expression of either GLI1 or Ha-ras in RK3E cells. To identify genes specific to the GLI1 transformation process, the expression profiles of cells transformed with GLI1 were compared with those from cells transformed with Ha-ras. Untransformed and transformed cells following transfection without drug selection were cloned, and gene expression profiles were established. We found 147 genes with altered expression levels from 4,608 UniGene clones, 30 as a result of stable GLI1 expression and 124 as a result of stable Ha-ras expression. Six genes were down-regulated by both GLI1 and Ha-ras expression. One gene was up-regulated by GLI1 and down-regulated by Ha-ras.

Genes whose expression was altered by GLI1 included cell cycle genes, cell adhesion genes, signal transduction genes, and genes regulating apoptosis. GLI1 consensus DNA-binding sequences were identified in the 5′ regions of cyclin D2, IGFBP-6 (IGF-binding protein 6), osteopontin, and plakoglobin. Gel shift analysis confirmed the ability of GLI1 protein to bind these sequences. Up-regulation of cyclin D2 and down-regulation of plakoglobin were also demonstrated in GLI1-amplified human tumor cells. Thus, cellular transformation by the human oncogene GLI1 proceeds via multiple downstream pathways.

EXPERIMENTAL PROCEDURES

Preparation of Transformed Cell Lines—Rat kidney epithelial cells stably transfected with E1A (RK3E cells, American Type Culture Collection, CRL 1895) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, pen-
icin (100 units/ml), and streptomycin (100 g/ml). Both the GLI1 and Ha-ras genes have been shown to transform RK3E cells (18). The human GLI1 (pLTR-GLI1 or Ha-ras (pOHST1)) (19) expression constructs were introduced into rat kidney cells (RK3E) by liposome-mediated transfection and transformed foci formed in 1–2 weeks. Cell lines were selected for neomycin resistance for GLI1- and Ha-ras-transfected cell lines. The Ha-ras-transfected cell lines did not show significant morphological differences compared with the parental RK3E cells at low density, however, and formed foci when grown to confluence. Ha-ras-transformed cells showed greater morphological changes and grew in a more anchorage-independent manner (data not shown) compared with the parental RK3E cells and even at low cell density formed foci. Both GLI1- and Ha-ras-transfected RK3E cell lines showed increased growth rates compared with RK3E cells. Western blot analysis using RK3E cells transformed with GLI1 revealed a 150-kDa GLI1 protein band. GLI1 protein was not detected in RK3E cells or Ha-ras-transformed RK3E cells establishing the presence of a stable protein product from the integrated GLI1 gene (data not shown).

**PCR Amplification for Microarrays**—Rat UniGene clones (4,608) supplied by Research Genetics (Huntsville, AL) were PCR-amplified with a primer set including T7/T3 promoters as follows: 5'-TACGAATTTAAA-TACGACTCACTATA-3', 5'-AAGCTAATATACTCCTCTACTAAGGGG-3'. GAPDH and β-actin served as control genes and were amplified with a primer set including M13/T7 promoters as follows: 5'-CAGGAAAAACA-GCTATGAC-3', 5'-GTAATACGACTATAGGGG-3'. PCRs were carried out in 96-well plates with a 1.2-μl volume using a Peltier Thermal Cycler PTC-225 (MJ Research, Watertown, MA) including 1 μl of 10× PCR buffer, 0.5 μl of Taq DNA polymerase purified using a Centri-Sep Column (Princeton Separation, Adelphia, NJ), 250 μM each primer, 200 μM each dNTP, and 0.4 μl of each cDNA clone. The first cycle was 95 °C for 11 min, 55 °C for 1 min, and 72 °C for 1.5 min. The 2nd to 36th cycles were 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min. The final extension was 72 °C for 7 min. After the reactions, 7 μl of water and 17 μl of Me2SO were added to the reaction mixtures. All PCRs were analyzed with 2% agarose gel electrophoresis using EAGLE EYE II (Stratagene, La Jolla, CA).

**Preparation of cDNA Microarray**—The microarrays were produced on poly-l-lysine-coated slides with a spacing of 180 μm in the area of 18 × 36 mm using a 4-pin arrayer (Genomic Solutions, Ann Arbor, MI). Each cDNA was stamped from the 384-well plates directly without rehydration or concentration with Microcon YM-30 columns (Millipore, Bedford, MA). The RNA bands were detected by autoradiography. The probes prepared by random priming (Rediprime II, Amersham Biosciences) were added to the concentrated probe. The hybridization solution (11 μl containing 3.5× SSC and 0.35% SDS) was applied to the microarray under a 22 × 22-mm coverslip for 17 h at 65 °C. The slide was rinsed in 2× SSC, 0.1% SDS for 2 min, 0.2× SSC for 2 min and then 0.05× SSC for 2 min at room temperature. The slide was scanned with a ScanArray 5000 (General Scanning, Waterton, MA) to detect the two-color fluorescence hybridization signals. Because of potential bias produced by different incorporation rates between Cy3- and Cy5-dUTP, two slides were used for each of four independent RNA preparations. For one slide, fluorescent cDNA probes were prepared from mRNA from RK3E cells (Cy5-labeled) and GLI1- or Ha-ras-transformed RK3E cells (Cy3-labeled). The Cy5-dyes were conjugated with mRNA of GLI1- or Ha-ras-transfected RK3E cells (Cy3-labeled) and RK3E cells (Cy5-labeled). The procedure was performed using two independent clones obtained from two independent transfections for both GLI1 and Ha-ras transformed cells.

**Analysis**—Data reduction was done with the program Glems 2.0 (National Institutes of Health, Bethesda, MD). Each spot was defined by manual positioning of a grid of circles over the array image. Signal intensity was determined by subtraction of local background from the mean intensity. Normalization between the dyes was accomplished by normalizing each dye to mean intensities of all genes. The threshold was set at 50% of the mean (Cy3 intensity + Cy5 intensity) of all genes for each array. In the plot of log (Cy3 + Cy5) versus log (Cy3/Cy5) (20, 21), genes with a signal intensity below 50% of the mean of (Cy3 + Cy5) showed large variances of the Cy3/Cy5 ratio. By using this threshold, 1778 and 1823 genes were selected in GLI1-RK3E versus RK3E and Ha-ras-RK3E versus RK3E microarrays, respectively. Following the intensity normalization, the log values of GLI1-RK3E or Ha-ras-RK3E versus RK3E ratios were counted for each experiment, and the mean ratios of four slides in each microarray were calculated for the same gene. The mean (S.D.) of the log of the ratios, calculated as described above, for all genes that met the 50% threshold were 0.003 (±0.133) for GLI1-RK3E versus RK3E and −0.019 (±0.199) for Ha-ras-RK3E versus RK3E cells. Genes were identified as having significantly different expression levels by comparison with the mean ± 2 S.D. of GLI1-RK3E versus RK3E (0.003 ± 0.266) to evaluate differential gene expression with a common criterion between GLI1-RK3E versus RK3E and Ha-ras-RK3E versus RK3E. The distribution of gene expression of Ha-ras-RK3E versus RK3E cells was wider than that of GLI1-RK3E versus RK3E cells indicating that Ha-ras induces expression changes in more genes than does GLI1. We evaluated the variation induced by differences in dye incorporation and variation by cell batch and slide. The log of ratios of all genes for each slide was compared with the above mean ± 2 S.D. (0.003 ± 0.266) and scored as follows. Ratios < mean – 2 S.D. were scored −1; ratios > mean + 2 S.D. were scored +1 and others were scored 0. The score was summed for all slides, and genes with scores of 4, 3, –3, or −4 were selected. The ratio of mRNA level is expressed as GLI1-RK3E/RK3E or Ha-ras-RK3E/RK3E. Positive values mean that the mRNA level in GLI1- or Ha-ras-transformed cells is larger than that in RK3E cells; negative values mean that the expression of the gene in transfected RK3E cells is lower than that in GLI1- or Ha-ras-transformed cells. Sequence homology searches of genes identified in this way were done using GenBankTM data bases and the BLAST search program (NCBI). The criterion of homology was 85% sequence match without gaps over 100 base pairs of sequence (22).

**Production of Biotinylated GLI1 Fusion Protein**—Biotinylated GLI1 fusion protein was produced in Escherichia coli using the PinPointTM fusion protein protocol (Promega). The pinpoint vector allows the production of a 1106 base pair GluFusion construct was prepared by inserting the BamHI fragment of the human GLI1 cDNA (pK12 GLI1) into the BamHI site of the PinPoint Xa2 plasmid (Promega). For protein production, bacterial cultures were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside and incubated for 3 h. Bacteria were then harvested, sonicated, and cleared by centrifugation. The GLI1 aa 211–1106 and control proteins (protein from Promega PinPoint plasmid or biotinylated GLI1 aa 879–1106, which lacks the zinc fingers) in the clear lysate were purified using SoftLinkTM Avidin resin (Promega).

**Electrophoresis Mobility Shift Assays**—Annexially 160 ng of the purified GLI1 aa 211–1106 protein or control protein was mixed with 5 μl of 2× binding buffer (50 mM HEPES, pH 7.5, 50 mM KCl, 5 mM MgCl2, 1 mM diithiothreitol, 20% glycerol (w/v), 50 μM poly(dI-dC), and 10 μM ZnSO4), H2O, and 0 or 1 μl (2 pmol) of unlabeled competitor or mutant GLI1-binding site competitor oligonucleotide (16). The mixture was incubated at 4 °C for 10 min. 1 μl (40 fmol) of double-stranded 32P-labeled probes (human cyclin D2 sense strand 5′-GACTTCTGTCG-CGCCACACCACTTCTCGCCT-3′ and human cyclin D2 antisense strand 5′-GACTTCTCGACCTGACCG-3′), 5′-IgFBP-6 sense strand 5′-GACTCCCTGACATCCATGGCCAAAATCCCATGACTGAGGG-3′; rat osteopontin sense strand 5′-GACTTGTGGCTGCTGAGCCATCATCCATGACTGAGGG-3′; rat osteopontin antisense strand 5′-GACTTTTATATGTTCCGTGCTGAGCCATCATCCATGACTGAGGG-3′; human plakoglobin sense strand 5′-GACTCTTCATTGAAGAACCCACCCACG-3′ and human plakoglobin antisense strand 5′-GACTCTTCATTGAAGAACCCACCCACG-3′; 5′-GACTTCTGTCG-CGCCACACCACTTCTCGCCT-3′.
Gene expression profiles show distinct sets of genes were regulated by GLI1 and Ha-ras-transformed RK3E cells. Microarray results are shown using mRNA from RK3E/HL cell lines (left) and the results using mRNA from RK3E/H11001 Ha-ras cells (middle). GenBank accession numbers, species, and genes identified by BLAST and UniGene search are shown (right). The source of the sequence data was human (H), mouse (M), and rat (R).
strand 5’-GACTCTGGTGTGTCCTTTCAAATGAGA-3’; and mutant GLI-binding site sense strand 5’-GACTCTCCGAAAGAATTGGGACAATGATGTC3’ and mutant GLI-binding site antisense strand 5’-GACTGAACATCATTGTCCAATTCGGGAG-3’ were added, and the mixtures were incubated at 4°C for an additional 20 min. The samples were analyzed by electrophoresis, and bands were visualized by autoradiography.

RESULTS

Gene Expression Profiles Revealed That GLI1 and Ha-ras Regulate Distinct Sets of Genes—We performed microarray assays using the RK3E cell line transformed independently with two oncogenes (Figs. 1 and 2) and examined 4,608 rat UniGene sequences. Comparison of expression profiles from GLI1- and Ha-ras-expressing cells established a set of genes unique to GLI1-induced cell transformation. This method identified 147 genes whose levels of expression were significantly altered in the RNA obtained from RK3E cells with stable GLI1 expression or stable Ha-ras expression. Thirty genes were altered by stable GLI1 expression as follows: 11 were highly up-regulated (>3 S.D.), 4 were up-regulated (>2 S.D.), 5 genes were highly down-regulated (>3 S.D.), and 10 genes were down-regulated (>2 S.D.). A total of 124 genes were changed by stable Ha-ras expression as follows: 30 genes were highly up-regulated (>3 S.D.), 19 genes were up-regulated (>2 S.D.), 56 genes were highly down-regulated (>3 S.D.), and 19 genes were down-regulated (>2 S.D.). Only 7 genes had altered expression levels in both RK3E cells with stable GLI1 expression and RK3E cells with stable Ha-ras expression. Six of these were down-regulated by both GLI1 and Ha-ras expression, whereas one of these, serine dehydratase-2, was up-regulated by stable GLI1 expression and down-regulated by stable Ha-ras expression. This observation clearly suggests that the oncogenes GLI1 and Ha-ras depend on distinct sets of gene activities to transform the same RK3E cells.

Annotation—The gene expression profiles of known genes are summarized in Tables I and II. The altered genes are distributed among 8 functional categories including cell cycle genes, cell adhesion genes, signal transduction genes, genes regulating apoptosis, and a small group of gene clones of unknown function.

PTCH, a member of SHH-Patched signaling pathway, which is believed to be a downstream target of GLI1 based on genetic analysis (23), was induced by GLI1 as predicted. We also observed induction of cyclin D2, implicating GLI1 in the regulation of the cell cycle.

RK3E+GLI1 cells also expressed a number of genes involved in cell structure, movement, and adhesion. Osteopontin and lysyl hydroxylase genes were up-regulated, whereas embigin and plakoglobin genes were down-regulated. A gene involved in IGF signaling (IGFBP-6) was up-regulated. A number of transcription factor genes showed altered expression in the GLI1-transformed cells. Npdc1 (neural proliferation differentiation and control gene 1) and TSC-22 (TGF-β-stimulated clone 22) are in this category. Known genes down-regulated by both GLI1 and Ha-ras were TSC-22, which is a target of the tumor suppressor TGF-β, and Tom1 (target of myb1).

Northern Blot Analysis Verifies the Microarray Assays—We performed Northern analysis to confirm the microarray data using probes to a number of the target genes identified by the microarray analysis (Fig. 2B). By using cDNA probes to the GLI1-regulated genes, significant increases in signal were seen for osteopontin, cyclin D2, H19, IGFBP-6, and PTCH, whereas significant decreases in signal were seen for TSC-22 and Tom1 as predicted by the microarray analysis.

Search for the Immediate Downstream Targets of GLI1—We have identified 30 genes regulated by the GLI1 oncogene. To determine whether these were direct downstream targets, we exploited the fact that GLI1 is a sequence-specific DNA-binding protein that interacts with the motif GACCACCCA. Among the
genes regulated by GLI1, we found five genes with 5’ sequences suitable for analysis. Of these, four genes contained putative GLI1-binding elements (Fig. 3). The putative GLI1-binding elements each show 1-base difference from the 9-bp consensus. It was shown previously that the HNF-3β enhancer, which contains 8 bp of matching sequence (GAACACCCCA), is a functional GLI1-binding site (16). A putative GLI1-binding motif (CACCACCCA) was found in the core promoter of human cyclin D2 (24). Osteopontin promoters of rat and mouse have a putative GLI1-binding site (GACCTCCCA). The motifs GTCCAC- (CACCACCCA) was found in the core promoter of human cyclin D2 and plakoglobin Northern data from human tumor cells. Genes with a ratio greater than 1.8 (RK3E/Hi1001) were up-regulated and 15 genes were down-regulated. This represents 0.65% of the genes examined, whereas 2.7% of the genes examined were altered by GLI1-binding sites in five genes for which appropriate sequence data were available that had altered expression levels in response to GLI1 sequence data were available that had altered expression levels in response to GLI1.

**DISCUSSION**

**GLI1 Activates a Unique Set of Genes during Cellular Transformation**—The GLI family of proteins are key mediators of SHH signaling in mammalian development. GLI1 is an oncogene expressed at high levels in many human cancers. GLI1 is a transcriptional activator, but the downstream targets of this gene, other than HNF-3β in mouse (16) and usg in Drosophila (25), have not been identified at a biochemical level. There are no proven human targets. Without knowing the range of genes regulated by GLI1, it will be difficult to understand its role in development or in cancer.

Here we show that the expression of 30 genes was altered by GLI1 expression in transformed RK3E cells. Among them, 15 genes were up-regulated and 15 genes were down-regulated. This represents 0.65% of the genes examined, whereas 2.7% of the genes examined were altered by Ha-ras expression. Importantly the gene expression profiles of GLI1 and Ha-ras did not overlap with the exception of seven genes regulated by both oncogenes. Furthermore, the genes activated by GLI1 were not those regulated by other oncogenes such as c-myc (26, 27). Thus, the expression profile for GLI1 transformation is specific and unique.

**GLI1-binding Elements Were Identified in the Promoters of Target Genes**—GLI1 has been proven to be a transcriptional activator, although convincing evidence of repressor activity is lacking. Nevertheless, we observed that a number of genes were down-regulated, although this may be the result of indirect effects of GLI1 expression. We examined the 5’ sequences for GLI1-binding sites in five genes for which appropriate sequence data were available that had altered expression levels in response to GLI1 expression. Functional GLI1 binding domains were found in four of them, one of which was down-regulated in response to GLI1 expression.

**GLI1 Regulates Cell Cycle Genes**—Recent evidence demonstrates that SHH opposes cell cycle arrest in epithelial cells (28). Because the GLI transcription factors mediate SHH sig-

**TABLE I**

| Gene categories | Induced | Repressed |
|-----------------|---------|-----------|
| **Cell division** | Cyclin D2 | Embigin |
| **Cell structure/adhesion** | Lysyl hydroxylase, Myosin 15/DRG2, Osteopontin | Plakoglobin (γ-catenin), rSmac2/LRT1-A (MHC-I) |
| **Signal transduction/growth** | H19, Patched, rIGF-binding protein 6 (rIGFBP-6) | Target of myb1 (Tom1), TSC-22, Plakoglobin |
| **Apoptosis** | | |
| **Transcription factors** | Neural proliferation, differentiation, and control gene 1 (npdc1) | TSC-22 |
| **Metabolism** | Palmitoyl protein thioesterase 2, Serine dehydratase (SDH2), Ubiquitin-specific protease 7 (USP7) | Acyl-CoA dehydrogenase, Cis-Golgi p28 |
| **Others** | Human clone DKPZp564B07 | Human clone KIAA0491, Human FLJ10514, Mouse clone 1–44 (growth suppressor) |
| **Repressed by both GLI1 and Ha-ras** | TSC-22, Tom1, FLJ10514, Smac21/R1T1-A |

**Human Tumor Cells with GLI1 Show Up-regulation of Cyclin D2 and Down-regulation of Plakoglobin**—By using probes for human cyclin D2 and human plakoglobin, Northern blots were performed on cells derived from two different human rhabdomyosarcomas (Fig. 5). The A673 rhabdomyosarcoma does not express GLI1, whereas RMS-13 is highly amplified for GLI1. The A673 cells do not express demonstrable levels of cyclin D2 by Northern blot but have abundant plakoglobin message. In the RMS-13 human rhabdomyosarcoma cells, there is abundant cyclin D2 and no plakoglobin RNA. Thus, cyclin D2 and plakoglobin Northern data from human tumor cells are consistent with the microarray results.
naling and GLI1 is the demonstrated oncogene in the family, it is reasonable to suggest that down-regulating GLI1 is important in cell cycle arrest. Based on our microarray results, we postulate that GLI1 regulates growth via several gene networks. GLI1 directly regulates cyclin D2 expression, and the human cyclin D2 promoter (24) contains a GLI1 binding consensus that we show interacts with human GLI1 protein. The human cyclin D1 and cyclin D3 promoters have similar elements that do not physically interact with GLI1 (data not shown). Cyclin D2 regulates the G1/S boundary of the cell cycle. The net result of this change in gene expression in response to GLI1 is to drive cells continuously through the cell cycle.

**TABLE II**

| Genes regulated by Ha-ras in RK3E+Ha-ras cells. Genes with a ratio greater than 1.8 (RK3E/H11001, R/C) are listed. Genes were classified according to known functions. |

| Genes                           | Induced                                                                 | Repressed                                                                 |
|---------------------------------|------------------------------------------------------------------------|---------------------------------------------------------------------------|
| Cell division                   | Cyclin D1                                                               | Annexin VI                                                                |
| Cell structure/adhesion         | Collagenase (UMRcase)/matrix metallocproteinase13 (MMP13) Osteonecetic | Bamaolan                                                                   |
|                                 |                                                                        | BMP-3b, BMP-4                                                              |
|                                 |                                                                        | Calponin                                                                  |
|                                 |                                                                        | Collagen type I α1                                                        |
|                                 |                                                                        | Collagen type I α2                                                        |
|                                 |                                                                        | Collagen type III α1                                                      |
|                                 |                                                                        | Collagen type V α2                                                        |
|                                 |                                                                        | Fibroblast tropomyosin 4 (TM-4)                                           |
|                                 |                                                                        | FilaminA α (FLNA)                                                         |
|                                 |                                                                        | Lipocortin/annexin III (Anx3)                                             |
|                                 |                                                                        | Nonmuscle myosin heavy chain-B                                            |
|                                 |                                                                        | Tropomyosin                                                               |
| Signal transduction/growth      | A-interferon inducible protein (p27-h)                                  | β-prime COP                                                               |
|                                 | Intracellular target for insulin and growth factors (PHAS-1)            | Frizzled homolog 1 (FZD1)                                                 |
|                                 | GADD 153                                                                | Frizzled homolog 2 (FZD2)                                                 |
|                                 | SAP kinase-3                                                             | GADD 45 γ                                                                 |
|                                 |                                                                        | Nogofoocen                                                                |
|                                 |                                                                        | Placental growth factor (Pfg)                                             |
|                                 |                                                                        | Prostaglandin F2α receptor regulatory protein                             |
|                                 |                                                                        | Protein tyrosine phosphatase (RAD2)                                       |
|                                 |                                                                        | Steroid-sensitive gene-1 protein (SSG-1)                                   |
|                                 |                                                                        | TSC-36                                                                    |
|                                 |                                                                        | Tom1                                                                      |
| Apoptosis                       |                                                                        | TSC-22                                                                    |
| Transcription factors           | Basic transcription element binding protein 2 (Bte2)                    | Breast cancer putative transcription factor (ZABC1)                        |
|                                 | Metallothionin-1 gene transcription factor/RNA polymerase II subunit    | BRMS/SNF 2a                                                               |
|                                 | Serum response factor                                                    | HES-5                                                                     |
| Metabolism                      | Asparagine synthetase                                                   | LIM protein CLP36                                                         |
|                                 | β-1,4-galactosyltransferase III                                         | LIM protein FHL1                                                          |
|                                 | Mitochondrial ATP synthase                                              | SRY-box-5 (Sox4)                                                          |
|                                 | coupling factor 6                                                        | Tepoisoeraser II                                                          |
|                                 | Mitochondrial F1-ATP synthase β-subunit                                  | CD81                                                                      |
|                                 |                                                                        | 3-Oxoadic CoA transferase (OXCT)                                          |
|                                 |                                                                        | β-glucuronidase                                                           |
|                                 |                                                                        | ER protein ERb20                                                           |
|                                 |                                                                        | Red-1                                                                     |
|                                 |                                                                        | Protein disulfide isomerase                                               |
|                                 |                                                                        | Squalene epoxidase                                                        |
|                                 |                                                                        | Squalene synthetase                                                       |
| Protein synthesis               | S3, S6, S10, S17, L12, L27a, L30, L37, L38, L39                          | Acidic ribosomal phosphoprotein P1                                        |
| Others                          | Antioxidant enzyme B166                                                  | Clone MNCh-2755                                                           |
|                                 | DKFZp547C176                                                             | FLJ10514                                                                  |
|                                 | DKFZp586KI123                                                            | HNRNP A2/B1                                                               |
|                                 | FLJ10483                                                                 | HSP 86–1                                                                  |
|                                 | HMC class 1b antigen (RT1.cl)                                            | HSPC148                                                                   |
|                                 | PTD9004                                                                  | Neuronal delayed rectifier K⁺ channel (K-V-4)                             |
|                                 | SNRPG                                                                    | NIP1-like protein (NipilA3)                                               |
|                                 |                                                                        | PAF-AH α1                                                                 |
|                                 |                                                                        | Prion protein                                                             |
|                                 |                                                                        | Sam21/RT1-A                                                               |
FIG. 3. GLI1-binding motifs in the 5′ region of GLI1 downstream targets. Potential GLI1-binding sites are underlined. The name of the gene is shown on the left (IGFBP-6, insulin-like growth factor-binding protein 6; HNF-3β, hepatocyte nuclear factor 3β). h, human; r, rat; m, mouse. HNF-3β sequence was from Ref. 16.

FIG. 4. Gel shift assays demonstrate GLI1 binds the 5′ regions. A, electrophoretic mobility shift assays demonstrated two shifted bands (arrows) with competition from the addition of 25–100 molar excess of non-radiolabeled human cyclin D2-specific oligonucleotide DNA. Control protein (Point protein or PinPoint fused to GLI1 aa 879–1106, lacking zinc fingers) or a mutant oligonucleotide DNA at 25–100-fold molar excess did not affect the mobility shift, indicating the specificity of the GLI1-cyclin D2 oligonucleotide interaction. B, IGFBP-6-specific oligonucleotide probe showed strong mobility shifts. 100-fold molar excess of non-radiolabeled IGFBP-6 oligonucleotide DNA completely abrogated the shifted bands. Mutant oligonucleotide (16) at 100-fold molar excess did not compete the shifted bands. C, similarly, osteopontin and plakoglobin probes resulted in specific shifted bands which were abrogated by 200-fold molar excess of non-radiolabeled oligonucleotide or mutant competitor.

FIG. 5. Northern blots of RNA from human rhabdomyosarcoma cells. The A673 human rhabdomyosarcoma cell line does not have GLI1 gene amplification. The RMS-13 human rhabdomyosarcoma cell line does have GLI1 amplification. Cyclin D2 is up-regulated and plakoglobin is down-regulated in GLI1 amplified RMS-13 cells. Thus, cyclin D2 and plakoglobin Northern data from human tumor cells are consistent with the microarray results.

GLI1 Regulates Signal Transduction Genes—The gene expression profiles showed that GLI1 expression regulates the activity of genes in several signal transduction pathways. Patched (PTCH), the transmembrane receptor of SHH, is up-regulated. Because PTCH inhibits smoothened, another transmembrane receptor, which activates GLI1, this establishes a negative feedback loop in the presence of SHH signaling. GLI1 down-regulates TGF-β-stimulated clones TSC-22 (a leucine zipper transcription factor). The pattern of expression during murine embryonic development of TSC-22 (38) is similar to that of GLI1. TSC-22 mediates TGF-β tumor suppressor function by activating apoptosis and repressing growth (39, 40). Because GLI1 represses TSC-22, loss of its tumor suppressor function may be an important consequence of GLI1 expression in malignant transformation.

Up-regulation of growth factor regulators was also discovered with this analysis. H19 and IGFB2 genes are imprinted and closely linked on the chromosome (41, 42). They can be up-regulated together in a variety of tumors (43). We observed increased H19 expression in GLI1-transformed RK3E cells. IGFBP-6 is an IGF2-binding protein whose function is not clearly understood. IGFBP-6 expression is stimulated by retinoic acid (44) and is inhibited by TGF-β1 (45). Its overexpression is also associated with several tumors. The up-regulation of H19 and IGFBP-6 by GLI1 expression may be involved in maintaining the proliferative state of cells and preventing cellular differentiation.

Combined Transcriptional Regulation by GLI1 Leads to Cellular Proliferation—In aggregate these results show that GLI1 provides strong cellular proliferation signals by transscriptional activation of cell cycle regulators, cell adhesion genes, growth factor regulators, and regulators of apoptosis (46). For example, activation of cyclin D2 forces cells through the cell cycle. Repression of embrygin and plakoglobin with induction of osteopontin reduces cell adhesion. Signaling changes in the SHH pathway itself by induction of PTCH, reduction of TSC-22, and changes in IGF signaling through increases in IGFBP-6 increase cellular proliferation and decrease apoptosis. In addition...
**Target Gene Regulation by GLI1**

![Diagram of GLI1 regulation](image)

**GLI1 expression alters regulation of apoptosis because plakoglobin expression is associated with apoptosis and TSC-22 mediates the apoptotic function of TGF-β (40).**

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