KLF9 Represses Cancer Stem Cell Transcription

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Background: Cancer cell stemness determines tumor propagation and malignancy by poorly defined mechanisms.

Results: The KLF9 transcription factor regulates cell fate and oncogenic pathways by repressing gene transcription in glioblastoma stem cells.

Conclusions: KLF9 inhibits glioblastoma cell stemness and tumor growth by directly repressing genes including ITGA6.

Significance: KLF9 and its transcriptional network are potential targets for regulating cancer stem cells and glial malignancies.

ABSTRACT

It is increasingly important to understand the molecular basis for the plasticity of neoplastic cells and their capacity to transition between differentiated and stem-like phenotypes. Kruppel-like factor-9 (KLF9), a member of the large KLF transcription factor family, has emerged as a regulator of oncogenesis, cell differentiation and neural development; however, the molecular basis for KLF9’s diverse contextual functions remains unclear. This paper focuses on the functions of KLF9 in human glioblastoma stem-like cells. We establish for the first time a genome-wide map of KLF9-regulated targets in human glioblastoma stem-like cells, and show that KLF9 functions as a transcriptional repressor and thereby regulates multiple signaling pathways involved in oncogenesis and stem cell regulation. A detailed analysis of one such pathway, integrin signaling, shows that the capacity of KLF9 to inhibit glioblastoma cell stemness and tumorigenicity requires ITGA6 repression. These findings enhance our understanding of the transcriptional networks underlying cancer cell stemness and differentiation, and identify KLF9-regulated molecular targets applicable to cancer therapeutics.

INTRODUCTION

Mounting evidence indicates that neoplastic stem-like cells, often referred to as cancer stem cells (CSCs), play an important role in several cancers, including glioblastoma (GBM), an aggressive malignancy in the adult central nervous system(1). CSCs are a minority population of slow cycling cells in tumors. They display various stem-like properties (hereby referred to as “stemness”) including long-term self-renewal and the capacity to generate phenotypically diverse hierarchical

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neoplastic progeny and stromal cells (2-4). A critically important characteristic of CSCs is their ability to efficiently propagate tumor xenografts that recapitulate essential phenotypes of the original tumor, such as tumor cell heterogeneity, invasiveness, and vascularity (2). Many CSCs, including glioblastoma stem cells (GSCs), are particularly resistant to chemotherapy and radiation (5-7). Thus, neoplastic cells displaying stem-like phenotypes are currently believed to contribute disproportionately to tumor growth patterns and recurrence after therapy (8).

Since CSCs and normal stem cells share phenotypic properties, it’s not unexpected that they also share signaling pathways (e.g. Notch, BMI1 and Hedgehog) that maintain their stemness and regulate their tumor propagating capacity (9-12). The self-renewal of non-neoplastic pluripotent stem cells is regulated by a pluripotency-driving transcription factor network, involving Oct4, Sox2, and Nanog (13,14), which also drives the stemness and tumorigenicity of CSCs (15-17).

Growing evidence is revealing that cancer cells are highly plastic with the capacity to dynamically and bidirectionally transition between more differentiated and more-stem-like phenotypes in response to contextual autocrine/paracrine signals (18,19). A more complete understanding of the gene regulatory networks that control neoplastic cell stemness is vital for understanding mechanisms behind the generation and maintenance of CSCs toward the goal of developing strategies for targeting CSC pools therapeutically.

One transcription factor family of particular interest in stem cell regulation is the family of Kruppel-like transcription factors (KLFs). KLFs consist of 17 zinc finger domain transcription factors that bind to GC or GT rich DNA regions to regulate transcription and thereby influence multiple biological events (20,21). KLFs are also involved in regulating stem cell self-renewal and differentiation. KLF4 is one of four Yamanaka transcription factors that, when ectopically expressed, reprogram somatic cells to pluripotent stem cells (22,23). Certain KLFs function as tumor suppressors and/or oncogenes depending on the cellular context. For example, the localization of KLF4 to the nucleus in early stage ductal carcinoma is associated with a more aggressive phenotype (24). KLF6 functions as a tumor suppressor that is mutated and/or suppressed in several cancers, including breast cancer, prostate cancer and GBM (24,25).

KLF9 (also known as basic transcription element binding protein, BTEB), a relatively unexplored KLF family member, is expressed in various tissues, most abundantly in the brain, kidney, lung and testis (26), and regulates a variety of cellular functions including stem cell self-renewal and differentiation. For example, KLF9 has been shown to regulate differentiation of intestinal cells and oligodendrocytes, and is also essential for late-phase neuronal maturation in the developing dentate gyrus (27,28). KLF9 has been linked to cancers as it is downregulated in colorectal cancer and endometrial carcinoma (29,30). KLF9 was also found to be induced during the differentiation of GSCs and enforced KLF9 expression inhibits GBM cell stemness, in part, by suppressing Notch 1 expression and its downstream signaling (31).

The genome-wide gene targets of KLF9 have not been identified, and the molecular mechanism underlying KLF9’s functional contributions to the regulation of cancer cell stemness and oncogenesis remains elusive.

Here, we use RNA-sequencing (RNA-Seq) and chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) to investigate genome-wide KLF9 binding sites and regulated genes in human GBM-derived neurospheres enriched for GSCs. This first comprehensive genome-wide analysis of KLF9 targets has revealed a significant role for KLF9 in regulating several pathways involved in oncogenesis and stem cell regulation.

**EXPERIMENTAL PROCEDURES**

**Reagents**

All reagents were purchased from Sigma-Aldrich. Stock of all-trans retinoic acid (RA) was prepared in DMSO and diluted to 1µM in cell culture medium as a working concentration. Doxycycline (Dox) was diluted to a concentration of 0.5 µg/ml in cell culture medium as a working concentration. In all the experiments, the final DMSO concentration was <0.1% and DMSO also had no demonstrable effect on neurosphere cultures. Laminin was diluted to 10 µg/ml as a working concentration.

**Cell Culture**

The human GBM neurosphere lines GBM1a (0913) and GBM1b (0627), originally derived and
characterized by Vescovi and colleagues (32) and used extensively by us (17,31,33), were maintained in serum-free medium containing EGF and FGF. Cells were differentiated in EGF/FGF-free medium with 1µM RA or 1% fetal bovine serum (32,34,35). Primary GBM neurospheres 551 (JHH551) and 612 (JHH612) were derived from clinical GBM specimens obtained at Johns Hopkins Hospital following a published method (32). All human materials were obtained and used in compliance with the Johns Hopkins Institutional Review Board.

**Lentiviral Vectors and Cell Infection**

N-terminal 3xFLAG-tagged KLF9 was constructed by high-fidelity PCR and cloned into pTRIPZ and pLEX vector (Thermo Scientific) using AgeI and MluI. ITGA6 was cloned into pLEX vector with AgeI and MluI. Lentiviral packaging followed second generation lentivirus packaging protocol using psPAX2 and pMD2.G vectors (Addgene). Cells were infected with lentivirus at MOI of 5 along with Transduc (System Biosciences) and selected with puromycin (1 µg/ml) for stable cell lines.

**Cell Adhesion and Migration Assay**

GBM neurosphere lines were infected with lentivirus. 24h after infection, cells were treated ± Dox for 96h to induce KLF9 expression. Primary GBM neurospheres were infected with KLF9, ITGA6 lentivirus or both. Cells were dissociated and plated on laminin-coated wells for 2-6h. Adherent cells were stained with crystal violet, dissolved with 2% SDS, and quantified spectrophotometrically at 550 nm using a Spectra MAX 340pc (Molecular Devices) plate reader. Results show relative adhesion measured after subtracting the background absorbance from all values.

Cell migration assays were performed using laminin-coated transwell chambers. GBM neurosphere lines were infected by ITGA6 lentivirus for 24h and cells were treated ± Dox for 48h to induce KLF9. Primary GBM neurospheres were directly infected with KLF9 lentivirus. Upper chamber medium consisted of neurosphere culture medium without EGF/FGF, and lower chamber medium consisted of DMEM medium with 10% FBS. After 24h, cells that had migrated through the filter were fixed and stained with Hoechst 33342 (Invitrogen). Migration was quantified by counting cells from eight random fields.

**Western Blot**

Western blot analyses were performed using the Odyssey Quantitative Western Blot System (LI-COR Biosciences) following manufacturer’s instructions. Primary antibodies were: anti-FLAG-HRP, anti-β-actin (Sigma-Aldrich), anti-integrin α6, anti-BMI1, anti-Sox2, anti-CD133 (Cell Signaling), and anti-Nestin, anti-Olig2 (Santa Cruz). Secondary antibodies were labeled with IRDye infrared dyes (LI-COR Biosciences).

**Immunofluorescence**

Neurosphere cells were collected by cytopin onto glass slides and fixed with 4% paraformaldehyde. The cells were immunostained with anti-FLAG (Sigma-Aldrich), anti-integrin α6 (Cell Signaling) antibodies and Hoechst 33342 nucleic acid stain (Invitrogen). Secondary antibodies were conjugated with Alexa488 or Cyanine Cy3. Immunofluorescent images were captured and analyzed using Axiovision software (Zeiss).

**Tumor Xenografts**

All animal protocols were approved by the Johns Hopkins School of Medicine Animal Care and Use Committee. For intracranial xenografts, SCID mice received 5,000 viable cells in 2 µl DMEM by stereotactic injection to the right caudate/putamen. Cell viability prior to implantation was determined by trypan blue dye exclusion. Mice were perfused with 4% paraformaldehyde and the brains were sectioned for histological analysis as previously described (31). Tumor size was quantified on H&E-stained coronal sections using computer-assisted morphometry (MCID software). Tumor volumes (V) were calculated using the formula V = ab²/2 (length (a) and width (b)).

**Flow Cytometric Assay**

Flow cytometric analysis was done on unfixed cells were stained with CD133/2(AC133)- PE (Miltenyi Biotec) or ITGA6-FITC (CD49f; BD Pharmingen) antibody following manufacturer’s protocol using a FACSCalibur (Becton Dickinson). Mouse IgG labeled with PE or FITC was used as control.

**RNA Isolation and RNA Sequencing**

Total RNA was extracted using the RNasey kit (Qiagen). 4 µg total RNA was subjected to library preparation using Illumina TrueSeq RNA Sample Preparation Kit v2 (Illumina). Indexed adapters were used in order to pool 4 cDNA libraries into
one sequencing reaction. Libraries were assessed using an Agilent Bioanalyzer (Agilent Technologies) to confirm that cDNA fragment sizes were between 200bp and 500bp in length. Sequencing was performed using Illumina HiSeq 2500 platform (Illumina).

**Quantitative Real-Time PCR**

Fold enrichment was calculated using the following formula: $2^{\Delta C_t}$ (Ct Flag IP - Ct Mouse IgG IP). The percentage of input was calculated using $100 \times 2^{(\text{adjusted input} - \text{Ct Flag IP})}$, where the adjusted input = Ct Input - (log2 of dilution factor). Ct, threshold cycle.

Primer sequences (5' - 3') for gene expression:

- **KLF9**, AACTGCTTTTCCCCAGTGTG, TCCCATCTCAAAGCCCATTA; RAP2A, TCTACAGCTCTGCTACCCAGCA,

- **TCGCCTTTTGAGCGACCATCTC; CAMK2G**, GACGACGTCCTGCTACCCAGCA,

- TCCAGCTCCTCTCCTAGAAGCCCATTA; CDC42, TGACAGATTACGACCGCTGAGTT,

- **GAGGTGCTTCAGAAGCCTGAGGAGG; MAPK3**, GGGCAAGGCTACCTTGGAGTCC,

- **GGAGACTGTAGGTAGTTTCGG; PAK4**, GATATTCAGCAACACTGTGCC,

- **AGGAATGGGGTCTTCAGCCAGCT; PIK3C2B**, TCTCTTCTTCAATGTCGAGTCC,

- **CCTCCTGAAACGAGCTGTGTCT; PIK3R2**, CTACGATTTAGCAAGCCGCTGAGT,

- GAGACGTCTAGGTTAGTCTTGGAG; MAP3K11, CTGGATGGCTCCTGAGGTTATC,

- **CACAGTAAGGCTGCCAGCAGAT; NOTCH3**, GATGATTCGGGACAACCTGCC,

- **GIIT1**, CCGAGGACTGAGGACACTG;

ChIP-seq primer sequences (5' - 3'):

- **RHOV KLF9 Binding Site**, CTCTGACTGAATGGGTCCAG,

- **TGCCTGCCTTTCCTCCTCC; TACEK2G KLF9 Binding Site**, GAGAGCGGAGGGAGGAGG,

- **GCGAGGGCGGCTCCGGG; SOS1 KLF9 Binding Site**, GAGAGGCTGGGCTCGGGACG,

- **GAGGAATCTTACCATGAATGCCC; HES1**, GGAAATGACAGTGAAGCACCTCC,

- **GGAATATGCAGTGAATGCCC; HEY2**, GCAGAGCAGCAGCCATCTTATG,

- **GAGGAGAATCTTACCATGAATGCCC; NUMBL**, CCTCCTCAGCTAGCTGAGCAGT;

- **CACAGTAAGGCTGCCAGCAGAT; PIK3R2**, ATGGCACCCTTTCTAGTCCGAGA,

- **CTCTGAAACGAGCTGTGTCT; PIK3C2B**, CCTCTTTCTTGTACGAGCTTCC,

- **GGATGAGACAGCACCCTCCAGGAGGACAG; CAMK2G**, GCAGACACCATAGGACCAAGC;

- **CAGGCTCAGCCATGAGCCACAGGAGGACAG; SOS1**, GCAGACACCATAGGACCAAGC;

- **GGATGAGACAGCACCCTCCAGGAGGACAG; SOS1**, GAGGATATTACGACCGCTGAGT,

- **GGGCAAGGCTACCTTGGAGTCC; MAPK3**, CTGGATGGCTCCTGAGGTTATC,

- **GCAGAGCAGCAGCCATCTTATG; HEY2**, GCAGAGCAGCAGCCATCTTATG,

- **GAGGAGAATCTTACCATGAATGCCC; HES1**, CGGAGGAGCTGGGCTCGGGACG,

- **GAGGAATATGCAGTGAATGCCC; HEY2**, GGAATATGCAGTGAATGCCC,

- **GGAATATGCAGTGAATGCCC; HEY2**, GCAGAGCAGCAGCCATCTTATG,

- **GAGGAGAATCTTACCATGAATGCCC; HES1**, CCTCCTCAGCTAGCTGAGCAGT;

- **CACAGTAAGGCTGCCAGCAGAT; PIK3R2**, ATGGCACCCTTTCTAGTCCGAGA,
Binding Site/BTE Site A, CCAGAGCCGGCGGGTTAAGGTG, GGGCCTGCTGACTCTCCTTCTA; ITGA6 No BTE Site 1, GCTTTTATGATATCTTGGCACAGTA, ITGA6 No BTE Site 2, GACTGGCTGATCCTCCTTTAGA, ATTTTTGTCATTGCCCCTCACTTT; NOTCH1 BTE Site, CGGGTCCCTCCTCCCCGGAG, NOTCH1 No KLF9 Binding Site, CAGCTGGGCCCTCGTGTTTC, NOTCH1 No BTE Site, CGGGTCCCTCCTCCCCGGAG, CAGCTGGGCCCTCGTGTTTC; RAP2A KLF9 Binding Site, GATCTGGGCGTGGAGGCGCG, GIT1 KLF9 Binding Site, GCTTCTGCAGGAAGCGGCGC, GIT1 KLF9 Binding Site, GCTTCTGCAGGAAGCGGCGC; Random Genomic Region 1, GTCTGTACTCCCAGCTACTC, Random Genomic Region 2, GTGTCAAAGCTCGGAACTG, GTTGCCAGGTGATCCCAAC; Random Genomic Region 3, CTGCACTTGGCCAATATTTC, CTGCACTTGGCCAATATTTC; Random Genomic Region 4, CTAGCGGCTCTTTCCCAC; Random Genomic Region 5, CTGGACCCCGGCTCTGAG, CTGGACCCCGGCTCTGAG; Random Genomic Region 6, CTGGACCCCGGCTCTGAG, CTGGACCCCGGCTCTGAG; Random Genomic Region 7, CTGGACCCCGGCTCTGAG, CTGGACCCCGGCTCTGAG; Random Genomic Region 8, CGACGTCTGCGCGGGTACC, RND2 KLF9 Binding Site, CTGGACCCCGGCTCTGAG, CTGGACCCCGGCTCTGAG; Random Genomic Region 9, CTGGACCCCGGCTCTGAG, CTGGACCCCGGCTCTGAG; Random Genomic Region 10, CGACGTCTGCGCGGGTACC, RAP2A KLF9 Binding Site, CTGGACCCCGGCTCTGAG, CTGGACCCCGGCTCTGAG.

RNA-Seq Analysis
The raw reads were aligned to reference human genome build hg18 using TopHat (36) with default parameters. For each gene, the number of reads aligned to its exons were counted and summarized into gene level counts by R bioconductor package GenomicFeatures (37) based on UCSC refFlat table for hg18. Normalization between samples was carried out by R package edger (38,39), which controls sequencing depth and RNA composition effects.

Reproducibility Plots
RPKM, namely reads per kilobase per million mapped reads, for each differentially expressed gene was calculated by R bioconductor package GenomicRanges (37). The heatmap was generated according to the count table with scaling across the samples for each gene. The log_2 fold change, log2((IP RPKM+1)/(Control RPKM+1)), for each gene in each cell line was calculated. The log_2 fold change for KLF9-GBM1b is plotted against that of KLF9-GBM1a.

Chromatin Immunoprecipitation (ChIP) and Sequencing
Cells were subjected to ChIP using MAGnify ChIP system (Invitrogen) following manufacturer’s protocol. KLF9 bound DNA was immunoprecipitated using mouse anti-FLAG M2 antibody (Sigma-Aldrich) and Dynabeads Magnetic Beads (Life Technologies). Mouse IgG served as the control. ChIP-enriched DNA was used for PCR and deep sequencing. ChIP-enriched DNA or input DNA (10ng each) was subjected to library preparation using ChIP-Seq DNA Sample Prep Kit (Illumina) following manufacturer’s protocol. Sequencing was performed using Illumina HiSeq 2500 platform (Illumina).

Peak Calling
The 50 bp long raw KLF9 ChIP-Seq reads were aligned to the reference human genome build hg18.
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using Bowtie (40) allowing at most two mismatches in the first 28 bp “seed” bases. KLF9 binding sites were called using CisGenome (41) with default settings by comparing the two IP samples against the two control samples.

Reproducibility Plots
The number of reads aligned to the peak regions by each of the four ChIP samples was counted by R bioconductor package GenomicRanges (37) and then normalized for the library size for each sample. For each peak region, its normalized counts in a given sample was further subtracted by its mean normalized counts across samples and then divided by its standard deviation, which gave the scaled binding intensity.

De novo Motif Discovery
The 150bp long sequences centered at the peak summits for the top ranked 500 peaks were extracted and fed as the input for de novo motif discovery algorithm of CisGenome. Ten motifs of varying length with mean motif length being 12 were searched simultaneously. To obtain the truly KLF9 enriched motifs, the occurrence rate of motif in the 150bp long sequences centered at the peak summits for all peaks was compared with its occurrence rate in control genomic regions. The control regions were randomly chosen to match the GC content and distributional properties of ChIP-Seq peak regions (42).

KLF9 Target Gene Detection
The differential gene expression detection was carried out by R bioconductor package edgeR (38,43,44) with tagwise dispersion at FDR 5%. A matched study design was used since the four samples came from two cell lines. The list of differentially expressed transcripts was further filtered if the absolute values of \( \log_2 \) fold changes for differentially expressed transcripts comparing the case vs control exceeded 0.8. KLF9 target genes were defined as differentially expressed transcripts with \( \geq 1 \) KLF9-binding peaks in the \(-20\) kb to \(+10\) kb window surrounding the transcription start sites (TSSs).

Pathway Analyses
Canonical pathway analysis was performed using Ingenuity Pathway Analysis (Ingenuity). The significance of association between KLF9 targets and a canonical pathway was measured using the ratio between the number of KLF9 targets in the pathway and the total number of molecules in the pathway database. A Fisher’s exact test was performed to determine the association between KLF9 targets and canonical pathways.

Bioinformatic and Statistical Analyses
See Supplemental Experimental procedures for detailed descriptions of ChIP-Seq and RNA-Seq analytical procedures. Statistical differences for other experiments were evaluated by the Student’s t test, Wilcoxon paired t test or ANOVA followed by Tukey multiple comparison tests as appropriate. All data are represented as mean value ± SEM. All results reported are representative of at least three independent replications.

RESULTS

KLF9 Expression and GBM Cell Stemness
We previously found that induction of endogenous KLF9 expression is required for the responses of GBM neurospheres to forced differentiation stimuli and that enforced KLF9 expression inhibits GBM cell stemness (i.e. self-renewal, multipotency, stem cell marker expression) (31). In order to identify genome-wide KLF9 targets linked to stemness regulation in GBM, we first established appropriate KLF9 induction conditions in these GSC models. Two human GBM neurosphere lines (designated as KLF9-GBM1a and KLF9-GBM1b) were engineered to express a doxycycline (Dox)-inducible KLF9 transgene with 3xFLAG tag (Figure 1A), and Dox treatment (0.5µg/ml) for 48 hours induced KLF9 mRNA expression ~28-fold (Figure 1B, Left). This KLF9 induction was comparable to the magnitude of endogenous KLF9 induction (10- to 12-fold) in response to two inducers of GSC differentiation (RA and serum) following withdrawal of growth factors (Figure 1B, Right). Ectopic KLF9 protein levels plateaued by 2 days of Dox treatment and quickly diminished after Dox withdrawal (Figure 1C). Next, we examined the effects of ectopic KLF9 induction on the expression of molecular markers and drivers of GBM cell stemness. The expression of various marker and drivers of GBM cell stemness including BMI1, Nestin, Olig2and Sox2 decreased in response to KLF9 induction (10- to 12-fold) in response to two inducers of GSC differentiation (RA and serum) following withdrawal of growth factors (Figure 1B, Right). Ectopic KLF9 protein levels plateaued by 2 days of Dox treatment and quickly diminished after Dox withdrawal (Figure 1C). Next, we examined the effects of ectopic KLF9 induction on the expression of molecular markers and drivers of GBM cell stemness. The expression of various marker and drivers of GBM cell stemness including BMI1, Nestin, Olig2and Sox2 decreased in response to KLF9 induction consistent with our previously reported findings (31) (Figure1D).These GSC models with biologically relevant KLF9 induction that inhibits GBM cell stemness are suitable for identifying
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Genome-wide KLF9 targets in human cancer models.

**KLF9 Gene Binding and Gene Expression Signatures**

A genome-wide analysis of KLF9 targets was performed by combining gene expression profiling using RNA-seq and mapping of transcription factor binding sites using ChIP-seq in GBM neurospheres with ectopic KLF9 induction (strategy outlined in Figure 2A). KLF9-GBM1a and KLF9-GBM1b cells were treated ± Dox for 48 hours after which RNA was isolated and subjected to cDNA library preparation and deep sequencing. Over 16 million cDNA reads were generated for each of four samples representing two biological replicates (KLF9-GBM1a and KLF9-GBM1b) with or without KLF9 induction. More than 82% of the reads aligned to the human genome. Consistency between two biological replicates was confirmed by heat map clustering (Figure 2B) and correlation assay (Figure 2C, correlation coefficient = 0.63). Of all the 24,524 RefSeq transcripts (18,275 genes), 1,550 transcripts (1,161 genes, 6.4%) were upregulated (FDR ≤ 5%, log2 (Fold Change) ≥ 0.8, Table S1) and 2,843 transcripts (2,092 genes, 11.4%) were downregulated (FDR ≤ 5%, log2 (Fold Change) ≤ -0.8, Table S2) in response to KLF9 induction (Figure 2D).

Genome-wide KLF9 binding sites were identified using ChIP-Seq in GBM neurospheres. Cellular chromatin bound by FLAG-tagged KLF9 was specifically precipitated using anti-FLAG M2 antibody and Dynabeads (Figure 2E) and the specific ChIP-enriched DNAs were further processed to produce libraries for ChIP-Seq. Over 150 million reads were generated from ChIP-enriched DNA libraries and input controls derived from two biological replicates. More than 71% of the reads aligned to the human genome. Using CisGenome (41), 31,261 KLF9 binding peaks were called at a false discovery rate (FDR) of 1% (Table S3). The ChIP intensities within KLF9 binding peaks were consistent for both biological replicates as shown by heat map clustering (Figure 2F) and correlation assay (Figure 2G, correlation coefficient = 0.875). The majority of KLF9 binding sites localized to the region -20 to +10kb around TSS (58.9%), the region -2 to +1kb around TSS (37.9%), intergenic regions (54.0%) and introns (35.9%). Binding sites localized less frequently to 5’-untranslated regions (7.3%), exons (10.9%) and 3’-untranslated regions (1.2%) (Figure 2H). In comparison, randomly selected genomic regions differed substantially from the KLF9 binding pattern (Figure 2H). Furthermore, KLF9-binding sites, but not randomly selected genomic regions, were found to be highly enriched around transcription start sites (TSSs) (Figure 2I). The top 500 KLF9 ChIP-Seq peaks as ranked by their FDR were used to perform de novo motif discovery analysis (41). The consensus sequence 5’-G/AG/T GGG C/T G G/T GGCN-3’ was identified as the most enriched KLF9 binding motif (Figure 2J). This motif closely resembles the motifs of two Sp1/KLF family members, KLF4 (5’-NGGG T/C G G/T GG-3’) and Sp1 (5’-GGGGNNGGGG-3’) (Figure 2K), identified using the transcription factor binding site profile database JASPAR (45).

**Analysis of KLF9-regulated Gene Targets**

KLF9 ChIP-Seq and RNA-Seq datasets were combined to establish gene targets directly regulated by KLF9 (i.e. genes differentially expressed in response to KLF9 induction and having one or more KLF9-binding peaks within 20kb to +10kb of their TSSs). The expression changes of KLF9-bound and KLF9-unbound genes are summarized in Figure 3A. Among the 2,092 genes downregulated by KLF9, 1,849 (88.4%) fulfilled these criteria (Table S4, designated as KLF9 downregulated targets). This number was significantly higher than that calculated by random gene sampling (the number of KLF9-bound genes found among 2,092 genes randomly selected 1,000 times from the human genome; Figure 3B, top panel, p-value = 0). In contrast, among the 1,161 upregulated genes, only 726 (62.5%) were directly bound by KLF9, a number indistinguishable from that calculated by random gene sampling (the number of KLF9-bound genes found among 1,161 genes randomly selected 1,000 times from the human genome, Figure 3B, bottom panel). These results indicate that the predominant role of KLF9 is to serve as a transcriptional repressor. Therefore, we focused the functional analyses described below exclusively on the 1,849 KLF9 downregulated genes (Table S6).
Gene function annotation and Ingenuity Pathway Enrichment Analysis (46,47) were performed using KLF9 downregulated targets. The top pathways enriched with KLF9 downregulated targets included those active in cancer regulation (e.g. molecular mechanisms of cancer, CXCR4 signaling, integrin signaling and notch signaling), stem cell pluripotency signaling (mouse embryonic stem cell pluripotency) and signaling in neuron development (axonal guidance signaling, CREB signaling and semaphorin signaling in neurons) (Figure 3C; Table S5).

A subset of the KLF9 downregulated targets selected from pathways highly ranked by IPA (e.g. molecular mechanisms of cancer, CXCR4 signaling, Integrin signaling, Notch signaling) was further validated by qPCR in KLF9-GBM1b neurospheres and low-passage primary GBM neurospheres (GBM 551) (Figure 4A). The validated genes include Notch pathway members NOTCH1, PSEN2 and NUMBL; CXCR4 (encoding a chemokine receptor that contributes to cytoskeletal organization and mediates metastasis in various cancers) and CAMK2G (encoding a serine/threonine protein kinase associated with the proliferation, resistance and survival of cancer cells). Integrin signaling, which modulates multiple cellular processes including cell adhesion and migration, tumor cell invasion and cell stemness (48,49), was one of the top ranked and validated pathways enriched in KLF9 downregulated targets. ITGA6, encoding integrin α6 receptor subunit, was downregulated up to 89% following KLF9 induction (Figure 4A). Other downstream components of the integrin signaling pathway, such as ARPC1B, CAPN5, GIT1 and MYLK, were also validated to be downregulated following KLF9 induction up to 88%, 74%, 72% and 71%, respectively (Figure 4A). We further tested the expression of KLF9 target genes in response to different levels of KLF9 induction as controlled by Dox concentration (Figure 4B). Dox treatment at 0.5µg/ml induced KLF9 11-fold, comparable to the magnitude of endogenous KLF9 induction in response to two inducers of GSC differentiation (RA and serum) (Figure 1B, Right).

This treatment inhibited the expression of 11 KLF9 target genes but not 3 control genes that were not identified as KLF9 targets. The KLF9 binding peaks near the TSS of 12 KLF9 downregulated targets as marked in Figure 4B were further validated by quantitative ChIP-PCR (Figure 5A). We also compared the fold enrichment of KLF9 binding peaks in 7 targets to control genomic regions located either 10 or 20kb upstream from individual KLF9 binding peaks (Figure 5B). Peaks identified by ChIP-Seq showed significantly higher enrichment than control regions. These validation results render credibility to the predictive value of our genome-wide datasets.

**Regulation of Integrin α6 Expression and Function by KLF9**

Integrin α6 is the most upstream component of the integrin signaling pathway that was identified to be highly regulated by KLF9. Also, among the integrin pathway genes, ITGA6 was found to be one of the most downregulated by KLF9 (Figure 4A) and the ITGA6 promoter was validated by ChIP-PCR to have one of the most highly enriched (~75-fold) KLF9-bound chromatin peaks (Figure 5B). One KLF9 binding motif (referred to as basic transcription element, BTE site), was identified in the human ITGA6 promoter at -396 bps relative to the TSS (Figure 6A). This site was found to be conserved in mouse ITGA6 promoter (data not shown). KLF9 binding to this ITGA6 promoter region was confirmed in KLF9-GBM1b neurospheres by ChIP-PCR. FLAG-KLF9 co-precipitated with ITGA6 promoter regions containing the BTE site (segment A and B) but not with control regions lacking a BTE site (segment C and D) (Figure 6A and 6B). BTE-containing segments A and B were enriched compared with control segments C and D (Fig. 6B, 16.4 and 67.8 fold enrichment for segments A and B vs. 4.3 and 0.9 fold enrichment for segments C and D, respectively).

The effects of KLF9 induction on the level of integrin α6 mRNA and protein were quantified in two GBM neurosphere lines and two low passage primary GBM neurosphere isolates. KLF9 inhibited integrin α6 mRNA levels in all cell cultures (Figure 6C). KLF9 induction also inhibited the expression of integrin α6 isoform A and B (50) (Figure 6D). We further examined the dynamics of ITGA6 expression in response to ectopic KLF9 expression controlled by Dox. ITGA6 expression levels changed inversely with KLF9 levels; ITGA6 expression decreased along with KLF9 induction and rapidly returned to baseline levels after Dox.
withdrawal (Figure 6E). The effect of KLF9 expression on the number of integrin α6-positive cells within neurospheres mirrored its effects on bulk-culture integrin α6 levels. KLF9 induction reduced integrin α6-positive neurosphere cells within neurospheres from 14.4% to 7.0% (Figure 6F).

**Integrin α6 Repression by KLF9 Inhibits GBM Cell Stemness and Tumorigenicity**

We asked if KLF9 modulates GBM cell behavior and stemness by regulating integrin α6 expression. To this end, we examined how KLF9 induction modulates GSC adhesion and migration on laminin, the integrin α6 ligand and an essential component of the perivascular niche that supports normal and neoplastic neural stem cells (51,52). KLF9 was induced in GBM neurospheres for 4 days and cells were then transferred to laminin-coated tissue culture substrata. Control cells rapidly attached and spread within 2-6 hours. KLF9 induction inhibited cell spreading (Figure 7A, left panel) and decreased cell adhesion by 25-71% (Figure 7A, right panel). We asked if enforced integrin α6 expression could rescue the cell adhesion defects induced by KLF9. Lentivirus vectors were used to express KLF9, integrin α6, or both in GBM neurospheres for 48-96 hours prior to assessing cell adhesion and spreading on laminin. Enforced integrin α6 expression partially rescued the cell spreading defect (Figure 7A, left panel) and abrogated KLF9’s ability to inhibit cell adhesion by approximately 71%, 48%, 64% in the three GBM neurosphere cultures (Figure 7A, right panel). Migration through laminin-coated transwell membranes was reduced in response to KLF9 induction by 62-86% (Figure 7B). Enforced expression of integrin α6 also rescued this KLF9-induced cell migration defect (Figure 7B).

Integrin α6 is highly expressed in various stem cells (48,53) and cell surface expression was found to be enriched in GSCs and to maintain GSC self-renewal and tumorigenicity (48). We hypothesized that KLF9 inhibits glioma cell stemness in part by repressing integrin α6 expression. KLF9 downregulated the expression of stemness regulators and stem cell markers in GBM neurospheres (Figure 7C). Enforced integrin α6 expression reversed KLF9-induced suppression of CD133, Nestin, Sox2, BMI1 and Olig2 expression (Figure 7C). These results support the conclusion that KLF9 regulates molecular markers and drivers of GSC stemness, in part, by repressing ITGA6.

We found previously that KLF9 induction reduces the growth of intracranial tumor xenografts established from GBM neurospheres and extends the survival of mice bearing xenograft tumors (31). We hypothesized that KLF9 reduces xenograft tumor growth in part by repressing integrin α6 expression. We examined the effects of expressing KLF9, integrin α6, or both on the growth of intracranial (i.c.) xenografts established from GBM-derived neurospheres. KLF9, integrin α6, or both were expressed in GBM neurosphere cells by lentivirus infection (Figure 8A) and cells were implanted to the brains of immunocompromised mice 48h after virus infection. Mice (n=4 for each group) were sacrificed 60 days post transplantation and coronal histological brain sections were examined for tumor size. Enforced KLF9 expression significantly inhibited tumor xenograft growth compared to control xenografts (tumor volume ± SEM (mm$^3$): 9.0 ± 2.9 vs 34.4 ± 6.1, p<0.01). Expressing integrin α6 alone, as an additional control, had no effect on xenograft growth. However, integrin α6 expression rescued tumor growth inhibition induced by KLF9 (Figure 8B-C).

**DISCUSSION**

Cancer cells are highly plastic in their ability to shift between more differentiated and more stem-like states. This phenotypic plasticity is dynamically controlled by transcription factor networks that regulate cell multipotency, differentiation, and tumor propagating capacity through coding and noncoding RNAs (18). Identification of these regulatory networks is paramount to understanding cancer cell hierarchy, malignant progression and mechanisms of therapeutic resistance.

We previously reported that the KLF9 transcription factor inhibits glioma cell stemness, KLF9 induction was found to inhibit GSCs and the growth of GSC-derived tumor xenografts (31). The inhibitory effect of KLF9 on stemness diverges from the stemness-supporting effects of other KLFs, such as KLF2, 4 and 5 (13,14,54). The molecular mechanism underlying KLF9’s function in human cancer models had not been extensively examined. This current study...
establishes for the first time a genome-wide map of KLF9-regulated targets in a human GSC model. 31,261 genome-wide KLF9 binding peaks were identified in GBM neurosphere cells under biological conditions of KLF9-induced stemness inhibition. Amongst genes around these peaks, 1,849 gene targets were found to be directly downregulated by KLF9. The predictive value of these datasets is supported by our validation of KLF9-regulated gene expression and KLF9 binding sites in the KLF9 targets we identified. The majority of KLF9 binding sites were found to be located in intergenic regions and introns, and KLF9 binding peaks were found to be most enriched proximal to the TSS, patterns that support the transcriptional regulatory function for KLF9. Statistical analyses of the differentially expressed genes bound by KLF9 proximal to TSS showed that KLF9 functions primarily as a transcriptional repressor in the context of glioma neurospheres. This does not absolutely rule out the possibility that KLF9 has transactivating activity under defined biological contexts, particularly given the strong influence of biological context and genetic background on the gene regulatory activities including those of KLF family members (24, 55). A particularly relevant example is from Mitchell and DiMario (56) who found that KLF9 transactivated an FGFR1 promoter-reporter construct in human myoblasts but repressed the same reporter in differentiated myotubes. However, the capacity for these reporters to accurately mirror the regulation of endogenous FGFR1 expression in the same models was not addressed. KLF9 contains domains known to mediate complex formation with Sin3-histone deacetylase and with histone acetylase, highlighting its potential for gene repression and activation, respectively (57, 58).

Our is the first application of genome-wide de novo motif discovery to define the KLF9 binding DNA consensus sequence, 5’ G/A G/T GGG C/T G G/T GGCN 3’. This result is consistent with a previous report showing that KLF9 (also known as BTEB) binds to a GC-rich DNA sequence (26). We also confirmed the similarity of this KLF9 binding motif to the motifs of SP/KLF family members such as Sp1 and KLF4. KLF2, 4 and 5 have been shown to extensively share binding sites across the genome and collaboratively support the self-renewal of pluripotent stem cells (54). These and other SP/KLF family members share DNA binding motifs by virtue of highly conserved zinc-finger DNA-binding domains located in the carboxy terminal (21). The activating or repressing transcriptional effects of KLF family members can be attributed at least in part to their divergent amino terminal domains that are believed to mediate complex formation with other transcription co-regulators (21). Considerably more information is needed to fully understand mechanisms behind the collaborative or competitive interplay among SP/KLF family members and the context-dependent effects of this transcriptional network on cell stemness and malignancy.

Gene function annotation and pathway analyses revealed that signaling pathways relevant to cancer signaling, stem cell regulation and neural cell function are enriched in KLF9 downregulated gene targets. Integrin pathway members including ITGA6, ITGA9, ARPC1B, CAPN5, GIT1 and MYLK were found to be particularly enriched for KLF9 downregulated targets. Integrin signaling modulates various cellular processes, such as proliferation, migration and differentiation, through cell-cell and cell-matrix interactions (59). Integrins, a family of cell adhesion receptors, are upregulated in stem cells and believed to support cell stemness by mediating adhesive interactions between stem cells and their niches (60). Integrin α6, a subunit for two laminin receptors, is also highly expressed in several types of stem cells, including subventricular neural stem cells (NSCs) (61, 62) and facilitates NSC adherence to endothelial cells in the perivascular niche (62). Integrin α6 has also been identified as a marker for cancer stem cells including GSCs (48, 53). It has been proposed that integrin α6 is essential for the maintenance of GSC self-renewal and tumor-initiating cell phenotype, providing a potential target for anti-GBM therapies (48). Despite the involvement of integrin α6 in stem cell maintenance, little was known about the mechanisms that regulate its expression in cancer cells or how it regulates stem cell phenotypes. Within this context it is interesting that our genome-wide analysis identified KLF9-regulated targets involved with cell adhesion and migration mechanisms but not previously linked to cell stemness regulation. Examples include RAP2A, which codes for a Ras-related protein that
regulates cytoskeletal arrangement, adhesion and migration (63,64), ARPC1B, which codes for subunits of the Arp2/3 complex implicated in actin polymerization(65), and RhoV, which regulates expression of pro-invasion transcription factors(66). This suggests that KLF9 may be particularly important in regulating a transcriptional network directed at cytoskeletal, adhesive, and migratory function.

We present novel findings that specifically link the repression of integrin α6 expression to the mechanism by which KLF9 inhibits GBM cell stemness. Multiple complementary criteria were used to show that KLF9 induction inhibits integrin α6 expression by directly repressing ITGA6 promoter activity. These include KLF9-induced reductions in integrin α6 mRNA and protein, and the interaction between KLF9 and the ITGA6 promoter. The mechanistic relevance of integrin α6 repression by KLF9 is supported by the concurrent inhibition of laminin-dependent GBM neurosphere cell adhesion, cell migration, and cell stemness in response to KLF9 induction and their normalization by enforced integrin α6 expression. The capacity for enforced integrin α6 expression to reverse the KLF9-induced inhibition of stem cell markers supports that KLF9 regulates stemness in part by downregulating integrin α6 expression. GBM is a very hypervascular neoplasm and integrin α6-expressing GSCs preferentially localize to the perivascular niche that provides tropic signals and adhesive matrix proteins (e.g. laminin) to promote tumor cell stemness (67). Consistent with this trophic interaction, Lathia et al. found that silencing integrin α6 reduces the self-renewal and tumor-propagating capacity of GSCs (48). Further investigation into KLF9’s effects on the interaction between GSCs and perivascular niche could provide further insight into the mechanisms behind KLF9’s inhibitory role in GSC stemness and tumor suppression.

Two other signaling pathways, CXCR4 and Notch, which regulate malignancy and stem cell biology, were also among those significantly downregulated by KLF9. CXCR4 is a chemokine receptor with multiple functions, such as regulating the migration of stem cells and cancer cells (68,69). CXCR4 signaling is active in over 20 human tumors, including gliomas and contributes to tumor promotion in multiple ways (69,70). CXCR4 is highly expressed in GSCs (71) and was recently reported to facilitate the recruitment of GSC-derived pericytes to CXCL12-positive endothelial cells to support the tumor vasculature and tumor growth (3). In addition to our novel findings with KLF9, several other KLFs have been reported to regulate CXCR4 expression in cancer cells.KLF2 downregulates CXCR4 in oral cancer cells (72), while KLF5 has been shown to upregulate CXCR4 in prostate cancer (73). Notch signaling has a prominent role in maintaining stem cell phenotypes within a variety of developmental and neoplastic contexts (e.g. NSCs, mammary stem cells, leukemia, GBM and mammary carcinoma) by promoting self-renewal and inhibiting differentiating programs (74-76). Our current finding that KLF9 represses multiple Notch pathway components further supports our earlier discovery that KLF9 represses Notch1 expression and its downstream signaling (31). We now broaden these earlier findings by showing that KLF9 also downregulates NUMBL, a component of the Notch signaling pathway (77). Evidence supports a regulatory role for NUMBL in cell stemness. For example, NUMBL increases pluripotency marker expression in embryonic stem cells by synergizing with hedgehog signaling (77). In conclusion, we have identified gene targets directly regulated by KLF9 on a genome-wide scale in GBM-derived neurosphere model. We show that KLF9 inhibits GBM cell stemness and functions predominantly as a transcriptional repressor within this cellular context. KLF9 is shown to regulate multiple signaling pathways including those involved in oncogenesis, stem cell regulation, neuronal cell signaling, and integrin signaling. KLF9 is shown to inhibit GBM cell stemness and tumorigenicity and that these anti-tumor effects result from integrin α6 repression. These findings enhance our understanding of the transcriptional networks underlying cancer cell stemness and differentiation, and identify KLF9-regulated molecular targets applicable to cancer therapeutics.
REFERENCES

1. Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., Henkelman, R. M., Cusimano, M. D., and Dirks, P. B. (2004) Identification of human brain tumour initiating cells. Nature 432, 396-401

2. Visvader, J. E., and Lindeman, G. J. (2008) Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nature reviews. Cancer 8, 755-768

3. Cheng, L., Huang, Z., Zhou, W., Wu, Q., Donnola, S., Liu, J. K., Fang, X., Sloan, A. E., Mao, Y., Lathia, J. D., Min, W., McLendon, R. E., Rich, J. N., and Bao, S. (2013) Glioblastoma stem cells generate vascular pericytes to support vessel function and tumor growth. Cell 153, 139-152

4. Magee, J. A., Piskounova, E., and Morrison, S. J. (2012) Cancer stem cells: impact, heterogeneity, and uncertainty. Cancer cell 21, 283-296

5. Bao, S., Wu, Q., McLendon, R. E., Hao, Y., Shi, Q., Hjelmeland, A. B., Dewhirst, M. W., Bigner, D. D., and Rich, J. N. (2006) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 444, 756-760

6. Eyler, C. E., and Rich, J. N. (2008) Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 26, 2839-2845

7. Beier, D., Schulz, J. B., and Beier, C. P. (2011) Chemoresistance of glioblastoma cancer stem cells—much more complex than expected. Molecular cancer 10, 128

8. Persano, L., Rampazzo, E., Basso, G., and Viola, G. (2013) Glioblastoma cancer stem cells: role of the microenvironment and therapeutic targeting. Biochemical pharmacology 85, 612-622

9. Androutsellis-Theotokis, A., Leker, R. R., Soldner, F., Hoeppner, D. J., Ravin, R., Poser, S. W., Rueger, M. A., Bae, S. K., Kittappa, R., and McKay, R. D. (2006) Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 442, 823-826

10. Fan, X., Khaki, L., Zhu, T. S., Soules, M. E., Talsma, C. E., Gul, N., Koh, C., Zhang, J., Li, Y. M., Maciaczyk, J., Nikkhah, G., Dimeco, F., Piccirillo, S., Vescovi, A. L., and Eberhart, C. G. (2010) NOTCH pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts. Stem Cells 28, 5-16

11. Lessard, J., and Sauvageau, G. (2003) Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. Nature 423, 255-260

12. Heo, J. S., Lee, M. Y., and Han, H. J. (2007) Sonic hedgehog stimulates mouse embryonic stem cell proliferation by cooperation of Ca2+/protein kinase C and epidermal growth factor receptor as well as Gli1 activation. Stem cells 25, 3069-3080

13. Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G., Gifford, D. K., Melton, D. A., Jaenisch, R., and Young, R. A. (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122, 947-956
14. Wang, J., Rao, S., Chu, J., Shen, X., Levasseur, D. N., Theunissen, T. W., and Orkin, S. H. (2006) A protein interaction network for pluripotency of embryonic stem cells. *Nature* **444**, 364-368

15. Gangemi, R. M., Griffero, F., Marubbi, D., Perera, M., Capra, M. C., Malatesta, P., Ravetti, G. L., Zona, G. L., Daga, A., and Corte, G. (2009) SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. *Stem cells* **27**, 40-48

16. Zbinden, M., Duquet, A., Lorente-Trigos, A., Ngwabyt, S. N., Borges, I., and Ruiz i Altaba, A. (2010) NANOG regulates glioma stem cells and is essential in vivo acting in a cross-functional network with GLI1 and p53. *The EMBO journal* **29**, 2659-2674

17. Li, Y., Li, A., Glas, M., Lal, B., Ying, M., Sang, Y., Xia, S., Trageser, D., Guerrero-Cazares, H., Eberhart, C. G., Quinones-Hinojosa, A., Scheffler, B., and Laterra, J. (2011) c-Met signaling induces a reprogramming network and supports the glioblastoma stem-like phenotype. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 9951-9956

18. Li, Y., and Laterra, J. (2012) Cancer stem cells: distinct entities or dynamically regulated phenotypes? *Cancer Res* **72**, 576-580

19. Marjanovic, N. D., Weinberg, R. A., and Chaffer, C. L. (2013) Cell plasticity and heterogeneity in cancer. *Clinical chemistry* **59**, 168-179

20. Kaczynski, J., Cook, T., and Urrutia, R. (2003) Sp1- and Kruppel-like transcription factors. *Genome biology* **4**, 206

21. Pearson, R., Fleetwood, J., Eaton, S., Crossley, M., and Bao, S. (2008) Kruppel-like transcription factors: a functional family. *The international journal of biochemistry & cell biology* **40**, 1996-2001

22. Takahashi, K., and Yamanaka, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676

23. Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B. E., and Jaenisch, R. (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* **448**, 318-324

24. McConnell, B. B., and Yang, V. W. (2010) Mammalian Kruppel-like factors in health and diseases. *Physiological reviews* **90**, 1337-1381

25. Narla, G., Heath, K. E., Reeves, H. L., Li, D., Giono, L. E., Kimmelman, A. C., Glucksman, M. J., Narla, J., Eng, F. J., Chan, A. M., Ferrari, A. C., Martignetti, J. A., and Friedman, S. L. (2001) KLF6, a candidate tumor suppressor gene mutated in prostate cancer. *Science* **294**, 2563-2566

26. Imataka, H., Sogawa, K., Yasumoto, K., Kikuchi, Y., Sasano, K., Kobayashi, A., Hayami, M., and Fujii-Kuriyama, Y. (1992) Two regulatory proteins that bind to the basic transcription element (BTE), a GC box sequence in the promoter region of the rat P-4501A1 gene. *The EMBO journal* **11**, 3663-3671
27. Dugas, J. C., Ibrahim, A., and Barres, B. A. (2012) The T3-induced gene KLF9 regulates oligodendrocyte differentiation and myelin regeneration. *Molecular and cellular neurosciences* **50**, 45-57

28. Scobie, K. N., Hall, B. J., Wilke, S. A., Klemenhagen, K. C., Fujii-Kuriyama, Y., Ghosh, A., Hen, R., and Sahay, A. (2009) Kruppel-like factor 9 is necessary for late-phase neuronal maturation in the developing dentate gyrus and during adult hippocampal neurogenesis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**, 9875-9887

29. Kang, L., Lu, B., Xu, J., Hu, H., and Lai, M. (2008) Downregulation of Kruppel-like factor 9 in human colorectal cancer. *Pathology international* **58**, 334-338

30. Simmons, C. D., Pabona, J. M., Heard, M. E., Friedman, T. M., Spataro, M. T., Godley, A. L., Simmen, F. A., Burnett, A. F., and Simmen, R. C. (2011) Kruppel-like factor 9 loss-of-expression in human endometrial carcinoma links altered expression of growth-regulatory genes with aberrant proliferative response to estrogen. *Biology of reproduction* **85**, 378-385

31. Ying, M., Sang, Y., Li, Y., Guerrero-Cazares, H., Quinones-Hinojosa, A., Vescovi, A. L., Eberhart, C. G., Xia, S., and Laterra, J. (2011) Kruppel-like family of transcription factor 9, a differentiation-associated transcription factor, suppresses Notch1 signaling and inhibits glioblastoma-initiating stem cells. *Stem Cells* **29**, 20-31

32. Galli, R., Binda, E., Orfanelli, U., Cipelletti, B., Gritti, A., De Vitis, S., Fiocco, R., Foroni, C., Dimeco, F., and Vescovi, A. (2004) Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer research* **64**, 7011-7021

33. Sun, P., Xia, S., Lal, B., Eberhart, C. G., Quinones-Hinojosa, A., Maciaczyk, J., Matsui, W., Dimeco, F., Piccirillo, S. M., Vescovi, A. L., and Laterra, J. (2009) DNER, an epigenetically modulated gene, regulates glioblastoma-derived neurosphere cell differentiation and tumor propagation. *Stem cells* **27**, 1473-1486

34. Pollard, S. M., Yoshikawa, K., Clarke, I. D., Danovi, D., Stricker, S., Russell, R., Bayani, J., Head, R., Lee, M., Bernstein, M., Squire, J. A., Smith, A., and Dirks, P. (2009) Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. *Cell stem cell* **4**, 568-580

35. Campos, B., Wan, F., Farhadi, M., Ernst, A., Zeppernick, F., Tagscherer, K. E., Ahmadi, R., Lohr, J., Dictus, C., Gdynia, G., Combs, S. E., Goits, V., Helmke, B. M., Eckstein, V., Roth, W., Beckhove, P., Lichter, P., Unterberg, A., Radlwimmer, B., and Herold-Mende, C. (2010) Differentiation therapy exerts antitumor effects on stem-like glioma cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16**, 2715-2728

36. Trapnell, C., Pachter, L., and Salzberg, S. L. (2009) TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25**, 1105-1111

37. Carlson, M., Pages, H., Aboyoun, P., Falcon, S., Morgan, M., Sarkar, D., and Lawrence, M. GenomicFeatures: Tools for making and manipulating transcript centric annotations.
38. Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139-140

39. Robinson, M. D., and Oshlack, A. (2010) A scaling normalization method for differential expression analysis of RNA-seq data. *Genome biology* **11**

40. Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome biology* **10**

41. Ji, H., Jiang, H., Ma, W., Johnson, D. S., Myers, R. M., and Wong, W. H. (2008) An integrated software system for analyzing ChIP-chip and ChIP-seq data. *Nature biotechnology* **26**, 1293-1300

42. Ji, H., Vokes, S. A., and Wong, W. H. (2006) A comparative analysis of genome-wide chromatin immunoprecipitation data for mammalian transcription factors. *Nucleic acids research* **34**, e146

43. Robinson, M. D., and Smyth, G. K. (2007) Moderated statistical tests for assessing differences in tag abundance. *Bioinformatics* **23**, 2881-2887

44. Robinson, M. D., and Smyth, G. K. (2008) Small-sample estimation of negative binomial dispersion, with applications to SAGE data. *Biostatistics* **9**, 321-332

45. Bryne, J. C., Valen, E., Tang, M. H., Marstrand, T., Winther, O., da Piedade, I., Krogh, A., Lenhard, B., and Sandelin, A. (2008) JASPAR, the open access database of transcription factor-binding profiles: new content and tools in the 2008 update. *Nucleic acids research* **36**, D102-106

46. Xia, J., and Wishart, D. S. (2010) MetPA: a web-based metabolomics tool for pathway analysis and visualization. *Bioinformatics* **26**, 2342-2344

47. Thomas, S., and Bonchev, D. (2010) A survey of current software for network analysis in molecular biology. *Human genomics* **4**, 353-360

48. Lathia, J. D., Gallagher, J., Heddleston, J. M., Wang, J., Eyler, C. E., Macswords, J., Wu, Q., Vasanji, A., McLendon, R. E., Hjelmeland, A. B., and Rich, J. N. (2010) Integrin alpha 6 regulates glioblastoma stem cells. *Cell stem cell* **6**, 421-432

49. Takada, Y., Ye, X., and Simon, S. (2007) The integrins. *Genome biology* **8**, 215

50. Hogervorst, F., Admiraal, L. G., Niessen, C., Kuijkmans, I., Janssen, H., Daams, H., and Sonnenberg, A. (1993) Biochemical characterization and tissue distribution of the A and B variants of the integrin alpha 6 subunit. *J Cell Biol* **121**, 179-191

51. Lathia, J. D., Li, M., Hall, P. E., Gallagher, J., Hale, J. S., Wu, Q., Venere, M., Levy, E., Rani, M. R., Huang, P., Bae, E., Selfridge, J., Cheng, L., Guvenc, H., McLendon, R. E., Nakano, I., Sloan, A. E., Phillips, H. S., Lai, A., Gladson, C. L., Bredel, M., Bao, S., Hjelmeland, A. B., and Rich, J. N. (2012) Laminin alpha 2 enables glioblastoma stem cell growth. *Annals of neurology* **72**, 766-778

52. Shen, Q., Wang, Y., Kokovay, E., Lin, G., Chuang, S. M., Goderie, S. K., Roysam, B., and Temple, S. (2008) Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. *Cell stem cell* **3**, 289-300
53. Shinohara, T., Avarbock, M. R., and Brinster, R. L. (1999) beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 5504-5509.

54. Jiang, J., Chan, Y. S., Loh, Y. H., Cai, J., Tong, G. Q., Lim, C. A., Robson, P., Zhong, S., and Ng, H. H. (2008) A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nature cell biology* **10**, 353-360.

55. Tetreault, M. P., Yang, Y., and Katz, J. P. (2013) Kruppel-like factors in cancer. *Nature reviews. Cancer* **13**, 701-713.

56. Mitchell, D. L., and DiMario, J. X. (2010) Bimodal, reciprocal regulation of fibroblast growth factor receptor 1 promoter activity by BTEB1/KLF9 during myogenesis. *Molecular biology of the cell* **21**, 2780-2787.

57. Seo, S., Lomberk, G., Mathison, A., Buttar, N., Podratz, J., Calvo, E., Iovanna, J., Brimijoin, S., Windebank, A., and Urrutia, R. (2012) Kruppel-like factor 11 differentially couples to histone acetyltransferase and histone methyltransferase chromatin remodeling pathways to transcriptionally regulate dopamine D2 receptor in neuronal cells. *The Journal of biological chemistry* **287**, 12723-12735.

58. Zhang, J. S., Moncrieffe, M. C., Kaczynski, J., Ellenrieder, V., Prendergast, F. G., and Urrutia, R. (2001) A conserved alpha-helical motif mediates the interaction of Sp1-like transcriptional repressors with the corepressor mSin3A. *Mol Cell Biol* **21**, 5041-5049.

59. Barczyk, M., Carracedo, S., and Gullberg, D. (2010) Integrins. *Cell and tissue research* **339**, 269-280.

60. Chen, S., Lewallen, M., and Xie, T. (2013) Adhesion in the stem cell niche: biological roles and regulation. *Development* **140**, 255-265.

61. Qian, H., Tryggvason, K., Jacobsen, S. E., and Ekblom, M. (2006) Contribution of alpha6 integrins to hematopoietic stem and progenitor cell homing to bone marrow and collaboration with alpha4 integrins. *Blood* **107**, 3503-3510.

62. Staquicini, F. I., Dias-Neto, E., Li, J., Snyder, E. Y., Sidman, R. L., Pasqualini, R., and Arap, W. (2009) Discovery of a functional protein complex of netrin-4, laminin gamma1 chain, and integrin alpha6beta1 in mouse neural stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 2903-2908.

63. Prabakaran, I., Grau, J. R., Lewis, R., Fraker, D. L., and Guvakova, M. A. (2011) Rap2A Is Upregulated in Invasive Cells Dissected from Follicular Thyroid Cancer. *J Thyroid Res* **2011**, 979840.

64. Nancy, V., Wolthuis, R. M., de Tand, M. F., Janoueix-Lerosey, I., Bos, J. L., and de Gunzburg, J. (1999) Identification and characterization of potential effector molecules of the Ras-related GTPase Rap2. *The Journal of biological chemistry* **274**, 8737-8745.
65. Welch, M. D., DePace, A. H., Verma, S., Iwamatsu, A., and Mitchison, T. J. (1997) The human Arp2/3 complex is composed of evolutionarily conserved subunits and is localized to cellular regions of dynamic actin filament assembly. *J Cell Biol* **138**, 375-384

66. Guemar, L., de Santa Barbara, P., Vignal, E., Maurel, B., Fort, P., and Faure, S. (2007) The small GTPase RhoV is an essential regulator of neural crest induction in Xenopus. *Developmental biology* **310**, 113-128

67. Filatova, A., Acker, T., and Garvalov, B. K. (2013) The cancer stem cell niche(s): the crosstalk between glioma stem cells and their microenvironment. *Biochimica et biophysica acta* **1830**, 2496-2508

68. Miller, R. J., Banisadr, G., and Bhattacharyya, B. J. (2008) CXCR4 signaling in the regulation of stem cell migration and development. *Journal of neuroimmunology* **198**, 31-38

69. Liekens, S., Schols, D., and Hatse, S. (2010) CXCL12-CXCR4 axis in angiogenesis, metastasis and stem cell mobilization. *Current pharmaceutical design* **16**, 3903-3920

70. Ehtesham, M., Min, E., Issar, N. M., Kasl, R. A., Khan, I. S., and Thompson, R. C. (2013) The role of the CXCR4 cell surface chemokine receptor in glioma biology. *Journal of neuro-oncology* **113**, 153-162

71. Ping, Y. F., Yao, X. H., Jiang, J. Y., Zhao, L. T., Yu, S. C., Jiang, T., Lin, M. C., Chen, J. H., Wang, B., Zhang, R., Cui, Y. H., Qian, C., Wang, J., and Bian, X. W. (2011) The chemokine CXCL12 and its receptor CXCR4 promote glioma stem cell-mediated VEGF production and tumour angiogenesis via PI3K/AKT signalling. *The Journal of pathology* **224**, 344-354

72. Uchida, D., Onoue, T., Begum, N. M., Kuribayashi, N., Tomizuka, Y., Tamatani, T., Nagai, H., and Miyamoto, Y. (2009) Vesnarinone downregulates CXCR4 expression via upregulation of Kruppel-like factor 2 in oral cancer cells. *Molecular cancer* **8**, 62

73. Frigo, D. E., Sherk, A. B., Wittmann, B. M., Norris, J. D., Wang, Q., Joseph, J. D., Toner, A. P., Brown, M., and McDonnell, D. P. (2009) Induction of Kruppel-like factor 5 expression by androgens results in increased CXCR4-dependent migration of prostate cancer cells in vitro. *Molecular endocrinology* **23**, 1385-1396

74. Farnie, G., and Clarke, R. B. (2007) Mammary stem cells and breast cancer--role of Notch signalling. *Stem cell reviews* **3**, 169-175

75. Weng, A. P., Ferrando, A. A., Lee, W., Morris, J. P. t., Silverman, L. B., Sanchez-Irizarry, C., Blacklow, S. C., Look, A. T., and Aster, J. C. (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* **306**, 269-271

76. Kanamori, M., Kawaguchi, T., Nigro, J. M., Feuerstein, B. G., Berger, M. S., Miele, L., and Pieper, R. O. (2007) Contribution of Notch signaling activation to human glioblastoma multiforme. *Journal of neurosurgery* **106**, 417-427
77. Liu, L., Lanner, F., Lendahl, U., and Das, D. (2011) Numblike and Numb differentially affect p53 and Sonic Hedgehog signaling. *Biochemical and biophysical research communications* **413**, 426-431
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FOOTNOTES

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FIGURE LEGENDS

Figure 1. A KLF9 induction model for identifying genome-wide KLF9 gene targets.
(A) Lentiviral vector for Dox-inducible KLF9 expression (3FLAG-KLF9: KLF9 with N-terminal 3xFLAG tag; TRE: tet-inducible promoter; UBC: human ubiquitin C promoter; rtTA3: reverse tet transactivator; IRES: internal ribosome entry site; Puro: mammalian selectable marker). (B) KLF9 expression was quantified by qRT-PCR in KLF9-GBM1b cells after Dox treatment for 48h (left). GBM1b cells, cultured as neurospheres (control), were forced differentiated by FBS or RA for 3 days. KLF9 expression was quantified by qRT-PCR (Right). (C) KLF9-GBM1a cells were treated with Dox for 6 days and then passaged into Dox-free medium for 2 days. Whole cell lysates were collected and analyzed by FLAG western blotting. FLAG-KLF9 is induced by Dox and rapidly silenced following Dox withdrawal. (D) KLF9-GBM1a and KLF9-GBM1b cells were treated ± Dox for 4 days and subjected to immunoblotting against BMI, Nestin, Olig2 and Sox2. Fold expression normalized to Actin was shown below each lane. Data represents mean ± SEM; *: p < 0.01, t test or Dunnett test (B, right).

Figure 2. Genome-wide KLF9 gene targets identified by combining RNA-Seq and ChIP-Seq analysis.
(A) Strategy for identifying KLF9 gene targets in two GBM neurosphere lines with KLF9 induction after 48h Dox treatment. KLF9 gene targets represent genes that were bound by KLF9 and showed differential expression in response to KLF9 induction. (B) Heatmap of expression pattern for differentially expressed genes from RNA-Seq data. Gene expression was calculated by reads per kilobase per million mapped reads (RPKM). (C) Differentially expressed genes for KLF9-GBM1b cells were plotted against those for KLF9-GBM1a cells to show the reproducibility of RNA-Seq (Spearman's correlation coefficient (R)=0.63, p<0.001). (D) Volcano plot of all genes analyzed for differential expression (red dots: genes with FDR ≤ 0.05 and absolute log2 (fold change) > 0.8). (E) KLF9-GBM1a cells were treated with Dox for 48 hours. Anti-FLAG antibodies but not nonimmune IgG specifically precipitated 3xFLAG-tagged KLF9 from fragmented DNA-protein complexes. The FLAG A combination (anti-FLAG M2 antibody bound to Dynabeads) showed higher affinity than the FLAG B combination (anti-FLAG M2 antibody directly crosslinked to magnetic beads). (F) Heatmap of ChIP intensities for identified peaks in KLF9 ChIP-Seq data. The two control samples from both cell lines are clustered together and the two IP samples are clustered together by hierarchical clustering. (G) ChIP-Seq reproducibility as determined by scatter plot comparing peak intensities for KLF9-GBM1b and KLF9-GBM1a cells (Spearman's correlation coefficient (R)=0.875, p<0.001). (H) Genomic distribution of KLF9 binding peaks and randomly selected genomic regions. (I) Distribution around transcription start sites (TSSs) of KLF9 binding peaks and randomly selected genomic regions. (J and K) KLF9 binding motif identified using the top ranked 500 KLF9 binding peaks (J). KLF4 and Sp1 motifs are the most similar ones to our discovered
KLF9 Represses Cancer Stem Cell Transcription

Figure 3. Analyses of KLF9-regulated gene targets.
(A) The percentage of all genes that show differential expression following KLF9 induction and had one or more KLF9 binding peaks within -20 kb to +10 kb of their transcriptional start site (TSS).
(B) The enrichments of KLF9 binding to 2,092 randomly selected downregulated genes (top panel) or to 1,161 randomly selected upregulated genes (bottom panel) were determined 1000 times to generate null distributions (red bars). The dashed lines correspond to the number of KLF9 downregulated or upregulated genes identified by ChIP-Seq and RNA-Seq. Only the number of KLF9 downregulated genes differs significantly from the null distribution.
(C) Pathways enriched in KLF9 downregulated gene targets determined by Ingenuity Pathway Analysis. Pathways are ranked by –log(p-value) calculated by Fisher’s exact test. Ratio is calculated by dividing the number of KLF9 targets genes in the pathway with the total number of genes in the pathway (Ratio threshold: 0.05).

Figure 4. Validation of KLF9 downregulated gene targets.
(A) Ectopic KLF9 expression was induced in KLF9-GBM1b cells by Dox or in primary GBM neurospheres (GBM 551) by KLF9 lentivirus. After 48h, a subset of the KLF9 downregulated gene targets was analyzed by qRT-PCR (*: p<0.05).
(B) KLF9-GBM1b cells were treated with various Dox concentrations for KLF9 induction. The expression of 11 KLF9 targets and 3 control genes (ITGA5, ITGB5 and ITGAV) was analyzed by qRT-PCR. Red arrows indicate the Dox concentration consistently used in all the experiments. Data represents mean ± SEM.

Figure 5. Validation of KLF9 binding peaks.
(A) Representative tracks from ChIP-Seq results show the locations of KLF9 binding peaks proximal to the TSSs of KLF9 targets (Arrow heads: TSSs; Orientation of each transcript is marked by arrow).
(B) KLF9-GBM1b cells were treated with Dox for 48h and subjected to ChIP using FLAG and mouse IgG. The enrichment of KLF9 binding peaks marked by red boxes in B were determined by qPCR. (C) The fold enrichment of KLF9 binding peaks in 7 KLF9 targets was compared to control DNA regions -10kb or -20kb upstream of these peaks. Data represents mean ± SEM; *: p<0.01, t test or Dunnett’s test (C).

Figure 6. KLF9 downregulates Integrin α6 by promoter binding.
(A) Schematic of the human ITGA6 promoter with a KLF9 binding peak identified by ChIP-Seq (-25,000 to +3,000bp relative to TSS). Primers were designed for segments A-D.
(B) Dox-treated KLF9-GBM1b cells were subjected to ChIP using FLAG antibody and mouse IgG. Selective enrichment in qPCR (left panel) and conventional PCR (right panel) was detected for BTE-containing segments A and B but not for control segments C and D.
(C) ITGA6 expression was measured by qPCR in GBM neurosphere lines and primary GBM neurospheres after KLF9 induction for 48h.
(D) KLF9 was induced by Dox for 96h in GBM1a and GBM1b cells. Membrane protein extraction was subjected to immunoblotting against Integrin α6, which detected isoform A and B. Fold expression normalized to Actin was shown below each lane.
(E) KLF9-GBM1b cells were treated with Dox for 6 days, passaged to Dox-free medium for 6 days, and then passage again to Dox-containing medium for 6 days. Cells were collected on the days indicated and subjected to qPCR for ITGA6. ITGA6 expression levels changed inversely with Dox-induced KLF9 expression.
(F) KLF9-GBM1b cells were treated ± Dox for 96h and subjected to flow cytometry using anti-Integrin-α6-FITC antibody or isotype IgG control. Representative dot plots and the percentages of Integrin α6-positive cells are shown. Data represents mean ± SEM; *: p<0.01, t test.

Figure 7. ITGA6 repression by KLF9 regulates GBM neurosphere cell adhesion, migration and stem cell marker expression.
KLF9, ITGA6, or both were expressed in GBM neurosphere lines and primary GBM neurospheres. (A) KLF9-GBM1b cells were passaged onto laminin-coated culture substrata for 2-6h. Cell adhesion was evaluated by phase contrast microscopy (Left Panel) and quantitatively by spectrophotometric analysis of crystal violet-stained cells (Right Panel). KLF9 expression inhibited cell adhesion. Co-expressing Integrin α6 partially rescued cell adhesion inhibition by KLF9. (B) Cells were passaged to laminin-coated transwell membranes. Cell migration was evaluated 24 hours later by analyzing DAPI-stained cells. KLF9 expression inhibited transmembrane migration, which was rescued by Integrin α6. (C) Transfected KLF9-GBM cells were maintained in neurosphere growth medium for 96 hrs. Whole cell lysates were subjected to immunoblotting to assess the expression of FLAG-KLF9, Integrin α6, and the stem cell markers CD133, Nestin, SOX2, BMI1, and Olig2. Relative expression of each protein (normalized to Actin) is shown below each band. KLF9 inhibited the expression of stem cell markers and this response was partially rescued by enforced Integrin α6 expression.

Bar: 50 µm. Data represents mean ± SEM; *: p<0.05, **: p<0.01, ***: p<0.001, One-way ANOVA followed by Tukey test.

**Figure 8. Integrin α6 expression rescues KLF9-Induced growth inhibition of glioma xenografts.** GBM1b neurospheres were infected with lentivirus to express FLAG-KLF9, Integrin α6 or both. (A) Immunofluorescence of neurosphere cells shows the expression of ectopic FLAG-KLF9 and Integrin α6 (bar: 50 µm). (B) Equal numbers of viable cells were transplanted into the brains of SCID mice (n=4). H&E-stained coronal brain sections obtained from post implantation day 60 animals are shown (bar = 1 mm). (C) Quantification of tumor xenograft volumes shows that KLF9 induction inhibited xenograft growth and that integrin α6 expression rescued KLF9-induced anti-tumor response. Data represents mean ± SEM; *: p<0.01, **: p<0.001, One-way ANOVA followed by Tukey test.
Figure 1

A

B

C

D

KLF9 Represses Cancer Stem Cell Transcription
Figure 2

A. KLF9 Represses Cancer Stem Cell Transcription

B. Heatmap showing gene expression changes in KLF9-treated cells compared to controls.

C. Dot plot showing correlation between KLF9 expression and log fold change.

D. Box plot showing distribution of gene expression changes.

E. Western blot showing protein expression levels.

F. Heatmap showing gene expression changes in KLF9-treated cells compared to controls.

G. Scatter plot showing correlation between log IP intensity and log fold change.

H. Bar chart showing percentage of total peaks in KLF9-bound regions.

I. Histogram showing number of peaks across different genomic regions.

J. KLF9 binding motif.

K. Similar binding motifs for KLF4 and Sp1.
Figure 3

A

KLF9 Binding (65%)  No KLF9 Binding (35%)

% of All Genes

0 20 40 60 80 100

Upregulated  Downregulated  Not Differentially Expressed

B

Downregulated Genes

Frequency

0 50 100 150 200

Number of KLF9-Bound Genes

C

Upregulated Genes

Frequency

0 50 100 150 200 250

Number of KLF9-Bound Genes

KLF9-Bound Genes from Random Gene Sampling

KLF9-Bound Genes from Fused RNA-Seq and ChIP-Seq Datasets

-log(p-value)

0.0 2.5 5.0 7.5 10.0

Molecular Mechanisms of Cancer

Axonal Guidance Signaling

Colorectal Cancer Metastasis Signaling

Role of NFAT in Cardiac Hypertrophy

GNRH Signaling

CREB Signaling in Neurons

Semaphorin Signaling in Neurons

CXCR4 Signaling

Breast Cancer Regulation by Stathmin1

Ephrin B Signaling

Signaling by Rho Family GTPases

Mouse Embryonic Stem Cell Pluripotency

Melanocyte Development and Pigmentation Signaling

Myc Mediated Apoptosis Signaling

Endometrial Cancer Signaling

α-Adrenergic Signaling

Renin-Angiotensin Signaling

Integrin Signaling
Figure 4

A

KLF9-GBM1b

GBM 551

Normalized Fold Expression

Control = 1.0

Molecular Mechanisms of Cancer
CXCR4 Signalling
Integrin Signalling
Notch Signalling

B

KLF9

Normalized Fold Expression (Control = 1.0)

Dox (µg/ml)

0 0.02 0.1 0.2 0.5 2 10

Control Genes

Normalized Fold Expression (Control = 1.0)

Dox (µg/ml)

0 0.02 0.1 0.2 0.5 2 10

RND2
ADAM17
CDC42

APH1B
PKR2
PAK4

CAM2KG
SOS1
SOS2

ITGA5
ITGB5
ITGBAV

Dox (µg/ml)
Figure 7

A

Control KLF9 KLF9 + ITGA6

ITGA6

B

Control KLF9

ITGA6 ITGA6 + KLF9

C

KLF9 ITGA6

CD133

1.0 0.32 0.93 1.33

Nestin

1.0 0.02 0.78 0.49

Sox2

1.0 0.27 1.14 1.0

BMI1

1.0 0.40 1.09 0.98

Olig2

1.0 0.21 1.11 0.86

Actin
Figure 8

A  

ITGA6/DAPI  

FLAG/DAPI  

ITGA6  

KLF9  

KLF9+ITGA6  

KLF9+ITGA6  

B  

Control  

KLF9  

ITGA6  

KLF9+ITGA6  

C  

Intracranial Tumor Size (mm³)  

Control  

KLF9  

ITGA6  

KLF9+ITGA6  

*  

**
KLF9 Inhibits Glioblastoma Stemness through Global Transcription Repression and Integrin- α6 Inhibition

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