The transcription factor GLI1 cooperates with the chromatin remodeler SMARCA2 to regulate chromatin accessibility at distal DNA regulatory elements

The transcription factor GLI1 (GLI family zinc finger 1) plays a key role in the development and progression of multiple malignancies. To date, regulation of transcriptional activity at target gene promoters is the only molecular event known to underlie the oncogenic function of GLI1. Here, we provide evidence that GLI1 controls chromatin accessibility at distal regulatory regions by modulating the recruitment of SMARCA2 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 2) to these elements. We demonstrate that SMARCA2 endogenously interacts with GLI1 and enhances its transcriptional activity. Mapping experiments indicated that the C-terminal transcriptional activation domain of GLI1 and SMARCA2’s central domains, including its ATPase motif, are required for this interaction. Interestingly, similar to SMARCA2, GLI1 overexpression increased chromatin accessibility, as indicated by results of the micrococcal nuclease assay. Further, results of assays for transposase-accessible chromatin with sequencing (ATAC-seq) after GLI1 knockdown supported these findings, revealing that GLI1 regulates chromatin accessibility at several regions distal to gene promoters. Integrated RNA-seq and ATAC-seq data analyses identified a subset of differentially expressed genes located in cis to these regulated chromatin sites. Finally, using the GLI1-regulated gene HHIP (Hedgehog-interacting protein) as a model, we demonstrate that GLI1 and SMARCA2 co-occupy a distal chromatin peak and that SMARCA2 recruitment to this HHIP putative enhancer requires intact GLI1. These findings provide insights into how GLI1 controls gene expression in cancer cells and may inform approaches targeting this oncogenic transcription factor to manage malignancies.

The glioma-associated oncogene GLI1 was first identified in malignant glioma as a gene duplication (1). Subsequently it was found to be elevated in many tumor types including prostate (2), breast (3), and pancreas (4) and correlated to poor prognosis (5, 6). It has been demonstrated that GLI1 is a driver of oncogenesis by Fiaschi et al. (7), who demonstrated that GLI1 overexpression promotes tumorigenesis in murine mammary tissue. Mills et al. (8) showed the pivotal nature of GLI1 in tumorigenesis using the established pancreatic cancer mouse model driven by constitutively active KRAS. In this model, knockout of GLI1 disrupts tumor formation. Although there are established roles for GLI1 in several cancers, the mechanisms by which GLI1 operates to regulate gene transcription are not well-understood. We therefore approached the role of GLI1 in cancer by studying the gene transcriptional regulation mechanisms by which GLI1 controls gene expression.

The central role of transcription factors in converting upstream signaling to downstream gene expression depends on their binding to specific DNA sequences and recruiting co-factors that modify the chromatin landscape and the activity of RNA polymerase. The zinc finger domain of GLI1 recognizes a GC-rich nine-nucleotide sequence motif. The binding of GLI1 to that motif and the effect on gene regulation has been extensively characterized for the promoters of many target genes activated via Hedgehog (HH) (9), transforming growth factor β (10), and SULF2 (11) signaling.

Here, using a combination of a Gal4–UAS reporter system (12), mapping experiments, and immunoprecipitation studies, we identified the ATPase SMARCA2, a component of the BAF (SWI-SNF) chromatin remodeling complex (13), as a co-regulator of GLI1-mediated transcription. We found that ectopic expression of GLI1 increases chromatin accessibility using a MNase assay. Further, ATAC-seq assays supported the MNase data and indicated genome-wide changes in chromatin accessibility regulated by GLI1. It also showed that GLI1 is involved in regulation of chromatin opening predominately at sites distal to promoters. Similarly, SMARCA2 modifies chromatin access at sites distal to the promoters. We further evaluated the link between GLI1-mediated gene expression and these distal chromatin sites by integrating RNA-seq with the ATAC-seq data. We identified 32 genes differentially regulated by GLI1 with cis chromatin sites regulated by both GLI1 and SMARCA2. At the GLI1-regulated HHIP gene, we confirmed the presence of both GLI1 and SMARCA2 at the putative enhancer and a dependence upon GLI1 for SMARCA2 occupation at this site. Together, these results point to a transcription regulation mechanism in which GLI1 and SMARCA2 cooperatively alter chromatin accessibility at sites located distally to target genes.

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GLI1 and SMARCA2 regulate chromatin accessibility

GLI1 and SMARCA2 interact with GLI1 and promote its transcriptional activity

Results

GLI1-mediated transcription is regulated through multiple regions in its C-terminal transactivation domain (TAD)

We initially sought to identify which GLI1 domains have transcriptional activity using GLI1–Gal4–DBD chimeras (Fig. 1A) in HEK293G cells stably transfected with a Gal4–responsive UAS–luciferase reporter. Using this system, the C-terminal TAD region (539–1106 aa) of GLI1 was shown to possess very strong transcriptional activity, 68-fold higher than control expressing only the Gal4–DBD (Fig. 1B). In contrast, the regions N-terminal to the TAD, which encode the SUFU (Suppressor of Fused) binding, DNA binding, and nuclear localization motifs, had transcriptional activity similar to the control, indicating little to no activator function in these domains. Further mapping shows that smaller fragments within the 539–1106 aa TAD retain activity at reduced levels. In particular, the chimera including GLI1 aa 946–1106 retains ≈50% activity compared with the chimera with the full 539–1106 aa TAD, (Fig. 1B). Reporter assays in RMS13 and PANC-1 cells transiently transfected to express both the UAS reporter and GLI1–peptide/Gal4–DBD chimeric proteins similarly demonstrated that transcriptional activity of GLI1 is found in its TAD and within TAD subdomains (Fig. 1C and Fig. S1A).

Expression of individual chimeras and UAS–promoter binding for the above experiments was evaluated in HEK293G cells by Western blotting (WB) and ChIP–PCR assays, respectively. An antibody against Gal4 revealed the expression of chimeras aa 1–232, 232–394, 383–595, 594–787, and 787–946, whereas chimeras 539–1106 and 946–1106 were only detected using an antibody against the C terminus of GLI1 (Fig. S1B). All chimeras were detected, with 232–394 and 787–946 having the lowest signals, weakly above background. Using ChIP–PCR assays to detect binding to the UAS promoter, all chimeras were found to be enriched 10–40-fold over background using the Gal4 or GLI1 antibodies, except the aa 594–787 construct, which exhibited a much higher level of enrichment (Fig. S1C). Thus, all constructs appear to be expressed and bind to the UAS promoter. However, no conclusion could be reached regarding their relative expression because of the apparent variable recognition of the different chimeras by the Gal4 antibody in the WB and ChIP assays. Therefore, we cannot rule out the possibility that differential expression of the chimeras affects the results of the reporter assays. Nonetheless, our results indicate that all transcriptional activating function resides in different subdomains within the 539–1106-aa C terminus of GLI1.

SMARCA2 interacts with GLI1 and promotes its transcriptional activity

To define mediators of GLI1 activity through these newly identified TAD domains, we used the aforementioned Gal4–UAS reporter system to screen a panel of potential transcriptional co-regulators. We co-transfected the GLI1–TAD with the chromatin remodeling histone acetyl transferases SMARCA2 and SMARCA4 (14), the histone acetyl transferase P300/CBP-associated factor PCAF (10, 15, 16) and the transcription initiation complex subunit TAF9 (17). These co-factors have been shown to interplay with GLI1 and regulate GLI1 target expression (10, 14, 15, 18). Expression control for co-regulators was determined by WB (Fig. S1D). Interestingly SMARCA2 exhibited a 2.5-fold increase in GLI1 transcription activity above the GLI1–TAD alone (Fig. 1D). In contrast, the related ATPase SMARCA4 did not increase the transcriptional activity of the GLI1–TAD. PCAF and TAF9 increased transcriptional activity, but this increase was less than 50% above the GLI1–TAD threshold (Fig. S1E). SMARCA2 co-expressed with Gal4-chimera–containing fragments of the GLI1–TAD has significantly increased luciferase activity of the 946–1106-amino acid region of GLI1 (Fig. 1E), indicating a specific interaction of SMARCA2 with the region of GLI1 having the highest transcriptional activity. Finally, we investigated the functional interaction between full-length GLI1...
GLI1 and SMARCA2 regulate chromatin accessibility

GLI1 and SMARCA2 regulate chromatin accessibility at distal regions to gene transcription start sites

With the identification of SMARCA2 as a co-regulator of GLI1, we tested whether GLI1, like SMARCA2, can modulate chromatin accessibility using a MNase digestion assay. In triplicate assays, a time course showed, interestingly, that GLI1 overexpression caused a reduction in the overall nucleosome fragment sizes similar to the SMARCA2 overexpression (Fig. 3A). Expression of the transfected constructs was confirmed by WB (Fig. S2A). These results show that increased GLI1 expression enhances chromatin accessibility and led us to inquire what regions GLI1 and SMARCA2 co-regulate via alteration of chromatin density.

We assessed the genome-wide chromatin accessibility site alterations utilizing ATAC-seq. In triplicate experiments in RMS13 cells, GLI1 and SMARCA2 were knocked down with siRNAs. The efficacy of knockdown was confirmed for the ATAC-seq assays with the average mRNA reduction being greater than 85% for both siRNAs and corresponding strong protein reduction (Fig. S2B). The number of significantly changed peaks was statistically determined among three replicates comparing the siGLI1 or siSMARCA2 to their matched controls (siNTs) using the DiffBind program with a 0.1 FDR. There were 440 and 3875 significantly changed ATAC-seq peaks (156,745 and 158,446 total peaks) for GLI1 and SMARCA2 siRNA treatment, respectively. Only a few peaks, 5 of 440 and 28 of 3875, showed an increase in chromatin accessibility, with the majority showing a decrease in accessibility upon GLI1 and SMARCA2 knockdown. This is revealed in Fig. 3B, in which significantly changed peaks (in red) are predominately in the lower portion of the graph, and only a few red points occur in the upper portions of the graphs (indicating increased chromatin accessibility). These results, along with the MNase data, provide evidence that GLI1 and SMARCA2 predominantly enhance chromatin accessibility.

We next characterized the chromatin sites regulated by GLI1 and SMARCA2. First, we determined whether GLI1 sites are also regulated by SMARCA2. The average peak score for the 440 GLI1 ATAC-seq peaks was sorted and plotted on a heat map using Deeptools (20) (Fig. 3C, left panel). The same peaks and sort order were then used to show that SMARCA2 knockdown also reduces the chromatin accessibility at the same 440 sites (Fig. 3C, right panel). We then mapped the differentially regulated ATAC-seq peak locations relative to gene transcription start sites (TSSs) using GREAT (21) with basal parameters. Although regulatory regions can be very distal to their respective genes, most characterized GLI1-BSs have been in promoter regions (9, 22). Unexpectedly, we found most of the identified GLI1- and SMARCA2-regulated ATAC peaks to be outside the 5-kb promoter regions of the nearest cis-gene, in either direction (Fig. 3D). To compare the distribution of regulated peaks and SMARCA2 with a luciferase reporter containing an 8× GLI-binding site (GLI-BS) in the promoter (19). Following transient transfection in PANC-1 cells, SMARCA2 had little effect on transcriptional activity however, GLI1 alone stimulated activity ~8-fold (Fig. 1F). When SMARCA2 and GLI1 were expressed together, luciferase expression was increased dramatically to over 2-fold compared with GLI1 alone, indicating that SMARCA2 enhances full-length GLI1 transcriptional activity at a GLI1-BS containing promoter (Fig. 1F). Thus, this screening identifies SMARCA2 as a co-regulator of GLI1 transcriptional activity.

We next determined whether GLI1 and SMARCA2 have a physical interaction using co-immunoprecipitation (co-IP) assays. In RMS13 cells, the IP of GLI1 resulted in the co-IP of SMARCA2, for both co-expressed transfected proteins (Fig. 2A, left panel) and endogenous GLI1 and SMARCA2 (Fig. 2A, right panel). Further mapping of the GLI1–SMARCA2 interaction was performed by co-expressing full-length GLI1–HA with fragments of SMARCA2 tagged with FLAG in RMS13 cells. GLI1 was found to co-IP multiple regions of SMARCA2 including fragments 393–786 and 786–1179, with a possible weaker interaction with the bromodomain fragment (amino acids 1180–1590) of SMARCA2 (Fig. 2B). Interestingly, there is a stronger co-IP of the SMARCA2 fragments compared with the full-length proteins; this may be due to improved exposure of GLI1-binding regions or increased stability of the SMARCA2 fragments that facilitate the interaction. The lower diagram in Fig. 2B summarizes the domains of SMARCA2 that were tested. Together, these data indicate SMARCA2 is co-regulator of GLI1 activity at target genes, and multiple regions of SMARCA2 are important for SMARCA2–GLI1 interaction.
GLI1 and SMARCA2 regulate chromatin accessibility

Figure 3. ATAC-seq shows that GLI1 and SMARCA2 regulate chromatin density at positions distal to gene transcription start sites and enriched with GLI-BS. A, GLI1 and SMARCA2 increase chromatin accessibility. GLI1 and SMARCA2 were ectopically expressed for 48 h in HEK293G cells followed by MNase digestion over a time course. The control was the empty plasmid vectors used to express GLI1 and SMARCA2. A representative agarose blot with ethidium bromide detection is shown for 0-, 5-, and 10-min MNase digestion (n = 3). Molecular mass markers are indicated in kDa. B–F, changes in chromatin accessibility was evaluated through ATAC-seq in RMS13 cells. GLI1 and SMARCA2 were knocked down using targeted siRNAs and prepared for ATAC-seq. Chromatin accessibility changes were determined relative to the nontargeting siRNA control samples. Triplicate experiments were sequenced. B, The significantly changed peaks (log fold change) identified with the Differ package (FDR = 0.1) are shown in red on the MA plot (440 peaks for GLI1 and 3875 for SMARCA2). Chromatin accessibility is predominately reduced as shown by most points falling in the lower portion of the graph. C, The 440 significantly changed ATAC-seq peaks in the GLI1 data set are sorted by signal average using DeepTools computeMatrix (reference point, center) and visualized with plotHeatMap. The same 440 peaks and sorting order was applied to the SMARCA2 data set and visualized with plotHeatMap. The reduction of chromatin accessibility for the GLI1 knockdown also occurs in response to SMARCA2 knockdown. D, the distribution of the differentially regulated peaks by GLI1 and SMARCA2 is determined relative to gene TSSs using the GREAT package with basal parameters. E, the enrichment of GLI1 consensus sequences at the differentially accessible sites are determined by HOMER. Matrices from GLI2 and GLI3 ChIP experiments available in the HOMER package were used to identify the GLI-BS. F, the common differentially accessible sites (n = 67) between GLI1 and SMARCA2 were identified using bedtools intersect with minimum overlap set at 10%.

to all peaks, a comprehensive list was generated from the MACS2 peak lists by combining lists and merging overlapping peaks to generate a single, nonredundant list for each group, GLI1 and SMARCA2, which includes peaks in both the control and knockdown samples from all replicates. Mapping all the ATAC-seq peaks (Fig. S2C) showed a similar pattern, but the percentage of peaks in the 0–5 kb regions is higher at 7.5 and 7.3% compared with 2.3 and 1.3% for the differentially regulated peaks, GLI1 and SMARCA2, respectively. There is also an enrichment of GLI-BS in 66 and 13% of these respective

GLI1 and SMARCA2 regulated ATAC-seq peaks (Fig. 3E). Next, we examined the overlap between GLI1- and SMARCA2-regulated ATAC-seq peaks and identified 67 sites (Fig. 3F) with at least 10% overlap (average overlap, 515 bp; range, 197–940 bp). We explored whether there were additional chromatin regions in which GLI1 and SMARCA2 work in very close proximity. However, expansion of all the peak domains to include 500 additional bases in each direction (1000 bp added) did not add additional shared peak regions. Of the 67 co-regulated sites, 38 (56.7%) contain GLI-BS. Using GREAT with basal setting, 123 genes were identified to be cis-located (5’ or 3’) to these 67 overlapping peaks. Together, these data identify the 123 genes associated with sites in which GLI1 and SMARCA2 together regulate chromatin accessibility and identify these chromatin sites to be mostly outside the promoters, possibly enhancers.

We next investigated the association between GLI1 regulation of gene expression and chromatin accessibility modulated by GLI1 and SMARCA2 depletion. We focused on GLI1 as the key regulator of gene expression because SMARCA2 alone showed little gene expression change in our reporter assays (Fig. 1F). GLI1 was depleted using siRNA in RMS13 cells in triplicate. Genes differentially regulated (DE) by GLI1 siRNA compared with control siRNA were determined using DESeq2 with a 1.5-fold cutoff and a p value at less than or equal to 0.05. GLI1 mRNA and protein reduction was confirmed by RT-PCR and WB (Fig. S2B). There were 3666 significantly DE genes as shown in red in Fig. 4A; 49% of the DE genes were down-regulated, including the known GLI1 target genes PTCH1, PTCH2, and HHIP (9, 23). To focus on chromatin accessibility, we limited our search to genes near the 440 identified GLI1-regulated ATAC-seq peaks. As diagramed in Fig. 4B, 725 genes located cis to the 440 peaks were identified using GREAT. This list of 725 genes was intersected with the list of 3666 DE genes from RNA-seq to identify 174 DE genes in cis to the GLI1-regulated peaks.

We further characterized the ATAC-seq peaks associated with these 174 genes and found that 97% (376 of 387) of these peaks are located outside the 5-kb promoter regions of the associated genes (Fig. 4C), and 74% (126/174) are enriched with GLI-BS (Fig. 4D). In contrast, we found the presence of GLI1-BS in the promoter regions (2000 bp upstream and 100 bp downstream) of these 174 genes to be low at only 20% (35/174). In summary, we identified 174 GLI1-regulated genes that also possess GLI1-regulated chromatin sites enriched with GLI-BS. Finally, we found the intersection between the 174 GLI1-centric genes and the 123 genes identified near GLI1 and SMARCA2 co-regulated ATAC-seq peaks. This identifies 32 GLI1-regulated genes (Fig. 4E and Table 1) potentially controlled through enhancers in which GLI1 and SMARCA2 modulate the chromatin accessibility.

GLI1 and SMARCA2 are enriched at distal chromatin sites for HHIP, a GLI1-regulated gene

With the identification of putative regulatory regions where GLI1 and SMARCA2 both regulate accessibility, we looked for the presence of GLI1 and SMARCA2 at these sites to confirm
Figure 4. GLI1-regulated genes have distal putative enhancer regions with GLI-binding site enrichment. A, MA plot showing an approximately equal distribution of up and down-regulated genes following GLI1 knockdown as determined by RNA-seq analysis using DESeq2 at 1.5-fold change (n = 3). B, the differentially expressed genes located cis to GLI1-responsive ATAC peaks were identified. Using GREAT, 725 genes were identified cis to the GLI1 ATAC-seq peaks and then intersected with the 3666 DE GLI1 genes to identify 174 genes. C, the positions of the GLI1-responsive ATAC peaks were mapped relative to the transcription start site for the 174 genes using the GREAT package. D, the frequency of GLI1-BS in the promoters and the distal sites of the 174 genes was determined using the HOMER package to identify the GLI-BS by their matrices. E, the differentially expressed genes (32) with distal GLI1- and SMARCA2-responsive ATAC peaks (overlapping) were identified by comparison of the gene lists, 123 genes cis to overlapping GLI1- and SMARCA2-responsive ATAC peaks to the 174 DE genes near a GLI1-responsive ATAC peak.

Figure 5 shows the chromatin accessibility averaged for three independent ATAC-seq experiments in RMS13 cells and displayed as custom tracks in the UCSC genome browser for the HHIP locus (chromosome 4). Each knockdown has its own matched siNT from the same manufacturer as the specific siRNA. Using the three replicates for each condition, the DiffBind package identified the GLI1- and SMARCA2-regulated ATAC-seq peak (marked with an arrow) 360 kb upstream of the HHIP TSS (Fig. 5A). This peak contains a GLI1-BS and corresponds with ENCODE H3K4Me1 enrichment but not H3K27ac (Fig. S3), suggesting an inactive enhancer state. This peak region is also identified in ENCODE ChromHMM (24) to have a candidate enhancer chromatin state. With the identification of a putative regulatory region in which GLI1 and SMARCA2 both regulate accessibility, we looked for the presence of GLI1 and SMARCA2 at this HHIP putative distal regulatory region. Using ChIP–PCR in RMS13 cells (n = 4), we demonstrate GLI1 and SMARCA2 occupancy of this ATAC-seq–identified chromatin site with robust median percentage input close to the median levels of the nonspecific IgG control. Interestingly, the presence of SMARCA2 at the HHIP putative enhancer depends on GLI1 as shown by the 2.2-fold reduction in SMARCA2 presence in the GLI1 knockdown cells. In contrast, GLI1 occupancy was not dependent on SMARCA2 (Fig. 5B). RT-PCR demonstrated that HHIP gene expression was reduced 1.8- and 1.5-fold in response to GLI1 and SMARCA2 siRNA knockdown, respectively (Fig. 5C). These observations show that GLI1 and SMARCA2 localize to this distal chromatin site upstream of the HHIP gene and that GLI1 affects the recruitment of SMARCA2 and provide evidence that GLI1 and SMARCA2 work together to regulate this putative HHIP enhancer.

Discussion

GLI1 is a regulator of gene transcription implicated in promoting tumor formation and progression. Although multiple signaling mechanisms tightly control its activity in normal cells, it can be difficult to know which pathway to target in cancer and whether an alternative pathway will be used in developing drug resistance. Rather than controlling GLI1 at the level of upstream signaling, we propose that targeting GLI1’s ability to regulate transcription may provide a novel therapeutic approach for cancer. To do this, we need to understand how GLI1 interacts with its transcriptional co-activators. Therefore, we sought to identify active GLI1 domains and transcriptional co-factors that interact with these regions of GLI1. In this study, we identified multiple active domains within the GLI1–TAD and demonstrated the transcriptional regulation of the GLI1–TAD by the co-regulator SMARCA2. In addition, we identified the novel role of GLI1 as a regulator of chromatin accessibility at sites outside promoter regions.

Although the transcriptional activity of GLI1 had been previously determined to be a large portion of the protein at the C-terminal region, the important subdomains and co-factors for regulating its target gene expression are less well-defined. It is consistent in normal and cancer cell lines that the transcriptional activity of GLI1 is restricted to its TAD (539–1106 amino acids), within which we identified multiple active subdomains. A general pattern indicates higher transcriptional activity toward the more C-terminal end. This may be due in part to the acidic domain of GLI1 at residues 1038–1055. Acidic domains are important for protein–protein interactions, particularly in complexes.

We have identified the ATPase SMARCA2 as a co-activator of GLI1 activity that acts through specific domains in the GLI1–TAD. Although SMARCA4, PCAF, and TAF9 have been shown to interact with GLI1 in other publications (10, 14, 15, 17), our study provides novel insight into the roles of transcriptional co-factors in the regulation of GLI1 activity. Although SMARCA2 and SMARCA4 are very similar proteins with central roles in the BAF chromatin remodeling complexes, they differ in the genes they regulate (25) and the cells they are expressed in (26). We found that SMARCA2 but not SMARCA4 is able to stimulate the activity of the GLI1–TAD.
GLI1 and SMARCA2 regulate chromatin accessibility

Table 1
Differentially expressed gene cis to chromatin sites regulated by GLI1 and SMARCA2

| Gene      | Description                                      | GLI1 RNA Log2FC | GLI1 peak fold change | SMARCA2 peak fold change |
|-----------|--------------------------------------------------|------------------|------------------------|--------------------------|
| MMP11     | Matrix metalloproteinase 11                      | 0.72             | 2.02                   | 1.5                      |
| MRAP2     | Melanocortin 2 receptor accessory protein 2      | 0.58             | 1.79                   | 0.77                     |
| A4GALT    | α1A-Galactosyltransferase (P blood group)        | 1.80             | 0.88                   | 0.77                     |
| HHIP      | Hedgehog interacting protein                     | 1.72             | 1.73                   | 1.6                      |
| CHCHD10   | Coiled-coil–helix–coiled-coil–helix domain 10   | 1.10             | 2.02                   | 1.5                      |
| GORASP2   | Golgi reassembly stacking protein 2              | 1.07             | 0.83                   | 1.79                     |
| DCC       | DCC netrin 1 receptor                            | 0.84             | 1.28                   | 0.73                     |
| ZCHC8     | Zinc finger CCHC-type containing 8               | 0.74             | 0.01                   | 1.45                     |
| FSTL4     | Follistatin like 4                               | 0.23             | 1.77                   | 1.48                     |
| FRMD4A    | FERM domain containing 4A                       | 0.72             | 1.81                   | 1.66                     |
| NFIA      | Nuclear factor IA                                | 0.67             | 0.89                   | 1.57                     |
| LRCH1     | Leucine-rich repeats and calponin homology domain 1 | 0.58         | 1.88                   | 1.21                     |
| ACVR2A    | Activin A receptor type 2A                       | 0.61             | 2.22                   | 1.49                     |
| PDCC6IP   | Programmed cell death 6 interacting protein      | 0.62             | 2.36                   | 1.5                      |
| FBXO11    | F-box protein 11                                 | 0.68             | 1.85                   | 1.44                     |
| SPRY2     | Sprouty RTK signaling antagonist 2               | 0.73             | 1.99                   | 0.77                     |
| PRKG1     | Protein kinase C/GMP-dependent 1                 | 0.76             | 1.82                   | 1.88                     |
| MEF2C     | Myocyte enhancer factor 2C                      | 0.80             | 1.97                   | 1.68                     |
| CDC42E3   | CDC42 effector protein 3                         | 0.84             | 1.88                   | 0.15                     |
| DAR5      | Aspartyl-tRNA synthetase                         | 0.84             | 2.24                   | 0.16                     |
| FOXN2     | Forkhead box N2                                  | 0.89             | 1.85                   | 1.38                     |
| NOG       | Noggin                                           | 1.07             | 1.87                   | 1.44                     |
| CXCR4     | CXC motif chemokine receptor 4                   | 1.21             | 2.24                   | 1.78                     |
| ANXA3     | Annexin A3                                      | 1.29             | 2.05                   | 1.79                     |
| ELAVL2    | ELAV like RNA binding protein 2                  | 1.46             | 2                      | 1.33                     |
| OTUD1     | OTU deubiquitainase 1                            | 1.46             | 1.78                   | 1.54                     |
| GALNT10   | Polypeptide N-acetyl-galatosamyltransferase 10   | 1.51             | 2.21                   | 1.94                     |
| DCX       | Doublecortin                                     | 1.57             | 1.67                   | 1.77                     |
| KLHL31    | Kelch like family member 31                      | 1.62             | 2.11                   | 1.74                     |
| RN3       | Rho family GTPase 3                             | 1.68             | 1.63                   | 1.56                     |
| ADAMTS5   | ADAM metalloproteinase with thrombospondin type 1 motif 5 | 1.69   | 2.08                   | 1.78                     |
| SEMA3E    | Semaphorin 3E                                    | 1.82             | 2.21                   | 1.58                     |

In our study, TAF9 acted to generally increase transcription rather than specifically for the GLI1–TAD. In contrast, Yoon et al. (17, 18) identified the TAF9 component of the TFIID complex as interacting with the 1020–1091 region of GLI1 and activating GLI1 transcription activity. In addition, Bosco-Clement (27) reported that a full-length GLI1 plus TAF9 had enhanced transcription. However, in our study, we found TAF9 to behave as a general transcription factor but did not specifically enhance the transactivity of the GLI1–TAD in the context of the Gal4/UAS reporter assay. The discrepancy between studies might be explained by differences in GLI1 chimeric constructs, reporter assay systems, or cell types. In other studies, PCAF was shown to regulate—in a transforming growth factor β-dependent manner—the BCL2 promoter through a GLI1–SMAD–PCAF (10) axis. We found that PCAF expression did not enhance the transcriptional activity of the GLI1–TAD in reporter assays, suggesting that PCAF may require other regions of GLI1 for co-activation impact.

It was surprising to find that GLI1, like SMARCA2, increased the accessibility of chromatin in normal cells as shown in MNase digestion assays. Equally interestingly was that the regulated chromatin sites determined by ATAC-seq predominately occurred beyond regions considered as promoters. This finding suggests control of potential enhancer sites as a mechanism for gene regulation by GLI1, with cooperative regulatory activity by SMARCA2. We use the term “enhancer” in this paper in the general sense to describe regulatory regions outside the promoters. Follow-up studies (e.g. ChIP-seq) will be needed to fully define these sites as enhancers looking at the enrichment of H3K4me1 and H3K27ac marks in these regions and CRISPR-Cas9 experiments coupled with chromosomal capture assays to determine the functionality of these distal regulatory elements. The possibility of direct control is supported with confirmation of robust enrichment of GLI1-BS in these putative enhancers and a decrease in chromatin accessibility at overlapping loci between GLI1 and SMARCA2 knockdown. We identified GLI1-mediated DE genes with cis-located GLI1- and SMARCA2-regulated ATAC-seq chromatin sites. Although many transcription-controlling sites fall relatively close or consecutive to their regulated gene, many regulatory sites can be very distal, separated by several genes and even on different chromosomes. Therefore, it is probable that some identified altered chromatin sites regulate genes that we could not identify by proximity. There may be broader gene regulation events controlled through enhancer on enhancer effects, secondary gene regulation following primary perturbation of TFs or co-regulators, or through enhancer RNA promotion of transcription or complex stabilization. Secondary regulation events may explain the high number (3666 genes) of DE genes up- or down-regulated by GLI1 knockdown. To identify regulated genes located further than our cis-limited search, a chromatin conformation study would be beneficial. We identified an ATAC-seq site regulated by both GLI1 and SMARCA2 in a region distal to the known GLI1 target gene, HHIP. We confirmed by RT-PCR the siGLI1-dependent HHIP down-regulation and by ChIP–PCR the occupancy of GLI1 and SMARCA2 at the HHIP putative enhancer, and, interestingly, a dependence upon GLI1 for SMARCA2 occupation at the HHIP enhancer. This site is not currently listed in the GeneCards (28) registry. We also demonstrated that knock-down of either GLI1 or SMARCA2 decreased the expression of HHIP. To further characterize the role of GLI1 and SMARCA2
at additional ATAC-seq peaks, ChIP-seq for GLI1 and SMARCA2 would provide additional evidence for their interaction in chromatin accessibility. Taken together, our data show that GLI1 activity can be modulated by interaction with SMARCA2 and that the two proteins can cooperatively control chromatin accessibility at the HHIP putative enhancer and possibly other distal regulatory regions. The identification of this new interaction between GLI1 and SMARCA2 expands the molecular function of GLI1 as playing a role in distal regulatory elements and chromatin remodeling. Our study furthers the understanding of both GLI1- and SMARCA2-regulated mechanisms in cancer models and has potential relevance in normal cells where both proteins are expressed. Understanding GLI1/SMARCA2 interaction in both normal and disease states will increase our knowledge of the mechanisms underlying disease pathobiology and may provide potential targets to be exploited for cancer therapy.

Materials and methods

General cell culture

Standard incubation conditions of 37 °C and 5% CO2 were used in all experiments. Purchased medium was enriched with 10% fetal bovine serum. Dulbecco’s modified Eagle’s medium was used for HEK293G and PANC-1 cell lines. RPMI was used for RMS13 cells. The HEK293G cell line was kindly provided by Dr. Robert G. Roeder (Rockefeller University, New York, NY, USA). PANC-1 and RMS13 were obtained from the American Type Culture Collection (Manassas, VA, USA).

Plasmid preparation

Plasmids were constructed by subcloning DNA fragments obtained by PCR into pM (Clontech, Mountain View, CA, USA) for the Gal4–GLI1 constructs and p3×FLAG CMV14 (Sigma) for SMARCA2. The primers are listed in Table 2. GLI1–HA is in the PRK5 vector. SMARCA4, TAF9, and PCAF constructs are in 3× FLAG, PSG5 FLAG, and pCI FLAG vectors, respectively.

Transfection conditions

Reverse transfections were used in all experiments except where noted. In general, OptiMEM was added to an empty cell culture plate followed by plasmids or siRNA and finally XtremeGene (Roche) or RNAiMax (Invitrogen), respectively. XtremeGene was used in a 2:1 ratio (μl XtremeGene: μg plasmid).
**GLI1 and SMARCA2 regulate chromatin accessibility**

### Table 2

**Primers for DNA subcloning Gal4–GLI1 constructs**

| Amino acid region | Sense primer (5’ to 3’) | Antisense primer (5’ to 3’) |
|------------------|-------------------------|----------------------------|
| **GLI1**         |                         |                            |
| Full-length      | CCAAGCTTATTGCTAAGCTGAGACCCCA | CTAGTCTAGAGGAGCTAGTTGGATTTGG |
| 1–1234           | CCAAGCTTATTGCTAAGCTGAGACCCCA | CTAGTCTAGAGGAGCTAGTTGGATTTGG |
| 1232–393         | CCAAGCTTATTGCTAAGCTGAGACCCCA | CTAGTCTAGAGGAGCTAGTTGGATTTGG |
| 383–959          | CCAAGCTTATTGCTAAGCTGAGACCCCA | CTAGTCTAGAGGAGCTAGTTGGATTTGG |
| 539–1106         | CCAAGCTTATTGCTAAGCTGAGACCCCA | CTAGTCTAGAGGAGCTAGTTGGATTTGG |
| 594–787          | CCAAGCTTATTGCTAAGCTGAGACCCCA | CTAGTCTAGAGGAGCTAGTTGGATTTGG |
| 748–946          | CCAAGCTTATTGCTAAGCTGAGACCCCA | CTAGTCTAGAGGAGCTAGTTGGATTTGG |
| 946–1106         | CCAAGCTTATTGCTAAGCTGAGACCCCA | CTAGTCTAGAGGAGCTAGTTGGATTTGG |
| **SMARCA2**      |                         |                            |
| Full-length      | CAGTTGGATCTATGCTCAAGGCAAGGAGCCCA | GCTCTAGACCTTACCAATGTTGGATTTGG |
| 1–1344           | CGGATTAGTTATTGCTAAGGCAAGGAGCCCA | GCTCTAGACCTTACCAATGTTGGATTTGG |
| 1–785            | CGGATTAGTTATTGCTAAGGCAAGGAGCCCA | GCTCTAGACCTTACCAATGTTGGATTTGG |
| 786–1590         | GGAATTAGTTATTGCTAAGGCAAGGAGCCCA | GCTCTAGACCTTACCAATGTTGGATTTGG |
| 1–394            | CGGATTAGTTATTGCTAAGGCAAGGAGCCCA | GCTCTAGACCTTACCAATGTTGGATTTGG |
| 393–786          | CGGATTAGTTATTGCTAAGGCAAGGAGCCCA | GCTCTAGACCTTACCAATGTTGGATTTGG |
| 786–1179         | CGGATTAGTTATTGCTAAGGCAAGGAGCCCA | GCTCTAGACCTTACCAATGTTGGATTTGG |
| 1180–1590        | CGGATTAGTTATTGCTAAGGCAAGGAGCCCA | GCTCTAGACCTTACCAATGTTGGATTTGG |

RNAiMax was used at 1 µl/10 pmol siRNA. The components were mixed, distributed to cover the entire plate surface, and allowed to complex for 30 min. The appropriate cells numbers were added to each plate with dilution in their appropriate media, mixed, and placed in an incubator. Standard transfections were prepared in a tube with addition, in order, of Opti-MEM, plasmid, and X-tremeGene. After mixing and complex formation for 30 min, the mixture was added dropwise to cells plated the previous day, with fresh medium, and placed in an incubator. Expression cultures were harvested after 48 h, whereas siRNA knockdowns were collected after 72 h.

To measure GLI1 domain transcriptional activity, HEK293G, RMS13, and PANC-1 cells were reverse-transfected with 3.3E-6, 4.4E-6, and 2.0E-6 µg of the Gal4–GLI1 plasmids per cell respectively. For RMS13 and PANC-1 cells, the Gal4-luciferase reporter was transfected at 2.1E-6 and 2.0E-6 µg plasmid/cell, respectively. For Gal4–GLI1 and SMARCA2 co-transcriptional activity, HEK293G cells were reverse-transfected with plasmid DNA at 5.4E-6 µg of each DNA (10.8E-6 µg total) per cell. For co-IP in RMS13 cells, 4.25E-6 µg plasmid per cell for GLI1 and SMARCA2 were prepared for a reverse transfection. The co-IP in HEK293G cells had 1.67E-6 µg of each plasmid per cell reversed transfected. Knockdown of GLI1 and SMARCA2 in RMS13 cells for RNA-seq and ATAC-seq analysis was set up as reverse transfection with 6.68E-6 µg plasmid per cell.

### Luciferase assays

Transfected cells prepared for transcriptional activity analysis by luciferase detection were carefully washed twice with PBS, lysed in passive lysis buffer (Promega), frozen on dry ice, and stored at −20°C until analysis with Promega luciferase assay system per the kit instructions. All bioluminescent signals are normalized to total protein determined by the Bradford assay (Bio-Rad).

### Isolation of protein and WB

The cells for protein analysis and WB were trypsinized, centrifuged, and washed twice with PBS containing protease inhibitors, and the cell pellet was frozen on dry ice and stored at −20°C until processing and analysis. The cells were thawed on ice and suspend in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 0.5% Nonidet P-40) and 1× Complete protease inhibitor (Roche) for 10 min with occasional gentle mixing. The nuclei were disrupted with five strokes through a fine-gauge tuberculin syringe. The solution was centrifuged for 10 min at 4°C and 10,000 × g to pellet the debris. The supernatant was transferred to clean tube, total protein was quantitated by the BCA (Pierce) method, and the remaining solution was re frozen until PAGE and WB analysis. Loading was normalized to total protein. Mini-PROTEAN precast gels were purchased from Bio-Rad for PAGE protein separation. The separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) using a semidry transfer (Pierce 2G). The following antibodies were used for WB detection: GLI1 from Novus Biologicals NB600-600 or Cell Signaling 2643, SMARCA2 from Cell Signaling 11966, FLAG from Sigma 2814, HA from Sigma 11867423001, and PCAF from Abnova 12188.

Dilutions were 1:1000. Secondary antibodies were diluted 1:10,000. Digital images of the chemiluminescence signal were captured using the Bio-Rad Chemidoc MP or Thermo MYECL.

### MNase digestion

HEK293G cell were transfected with plasmid containing GLI1 or SMARCA2 and allowed to grow for 48 h. The cells were trypsinized then counted and aliquoted to 1.0E6 cells per reaction. The cells were washed and suspended in a MNase digestion buffer (20 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 1 mM CaCl₂) containing Complete (Roche) protease inhibitors. The samples were placed in a 37°C heating block for 5 min before the addition of 5 µl of MNase diluted 1:150. After 10 min of incubation with rotation, the digestion was terminated with the addition of a stop buffer (100 mM Tris-HCl, pH 8.1, 20 mM EDTA, 200 mM NaCl, 2% Triton X-100, and 0.2% sodium deoxycholate) and placed on ice. The DNA was isolated using a QiaAMP DNA mini kit (Qiagen), separated on a 1.5% agarose gel, and visualized with ethidium bromide.
**RNA isolation and quantitation**

The cells for mRNA analysis were trypsinized, centrifuged, thoroughly mixed in TRIZol (Invitrogen) or RLT buffer (Qiagen), frozen on dry ice, and stored at −80 °C until processing and analysis. RNA isolation was done following the protocol in the Qiagen RNeasy kit including the DNase I treatment and quantitated using the Nanodrop UV-Vis spectrophotometer (ThermoScientific), and cDNA was prepared using the high-capacity cDNA transcriptions kit (Applied Biosystems). The linear range for RNA conversion to cDNA was determined. Specific genes were amplified using the PerfeCTa SYBR Green SuperMix-Quanta, Bio-Rad CFX384 RT-PCR instruments and validated primers specific for the genes of interest. TBP and HPRT1 were used as housekeeping genes where appropriate. Expression primers are listed in Table 3.

**RNA-seq and ATAC-seq**

RMS13 cells were transfected with siRNAs targeting GLI1 (Qiagen FlexiTube Si03063641) or SMARCA2 (Invitrogen Stealth HSS110001) and the nontargeting controls from the same manufacture as the targeted siRNA (Qiagen 000102781, Invitrogen 12935300). siRNAs transfections were prepared as described under “Transfection conditions.” Triplicate experiments were prepared independently.

The RNA was isolated following the protocol in the Qiagen RNeasy kit including the DNase I step and submitted to the Mayo Clinic Genomic Analysis Core. The unstranded mRNA library was prepared using the Illumina kit v2 and sequenced (paired-end) on the Illumina HiSeq 4000 to a depth of 30 million reads. The reads were aligned to the genome. Sequencing was on an Illumina HiSeq 4000 to a depth of 50 million reads/sample. The reads were aligned to the genome. Clean up was done using the Zymo DNA clean and concentrator kit (Zymo Research). Clean up was prepared with 5 cycles of PCR amplification with the NEB–Complete and transferring to a clean tube. The cells were pelleted by centrifugation, suspended in ChIP lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% Nonidet P-40, 20 mM Tris, pH 8.1, 1× Complete) for 20 min followed by centrifugation to pellet the nuclei. The nuclei were suspended in nuclear lysis buffer (150 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, 1× Complete). Chromatin was sheared with sonication for 32 cycles (30-s on/off cycles) in a Diogenade Biorupter 300. After the shearing check, the samples were centrifuged to remove the debris, and the supernatant was transferred to a clean tube. The samples were then equally aliquoted with 2 E 6 cells per IP, diluted 1:10 with dilution buffer (0.01% SDS, 1% Triton, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167 mM NaCl, 1× Complete), and incubated with 2 μg of IgG control or a specific antibody overnight with slow rotation at 4 °C. Dynabeads Protein A/G (Thermo Fisher) were washed one time with dilution buffer, and 30 μl were added to each sample and allowed react for 4 h with slow rotation at 4 °C. The beads were gently washed three times with dilution buffer, and the DNA was eluted with 100 μl of elution Buffer (26.4 mM Tris-HCl, 0.01% NaOH, 0.01% HCl, 200 mM NaCl, 1% SDS) plus 1 μl of proteinase K and incubation for 2 h at 65 °C and 95 °C for 10 min. DNA was purified using a DNA purification spin column kit (Qiagen). Extracted DNA was diluted 1:20 with water and PCR-amplified with validated ChIP primers and SYBR Green detection; Gal4 UAS domain forward, GCATGCGATATTGCGCGAC, and reverse, AGATGAGACGACACTCCGAGTTG; and HHIP putative enhancer (Chr 4: 145206838–145207375) forward, TTGGATAAGGACTGATGAGATCC and reverse, ATGTGCCATAGACAGACCC.

### Table 3

| Gene | Forward | Reverse |
|------|---------|---------|
| GLI1 | TGCTTGATACCCCTCCCGGA | GCGATCTGATGATGATGAGATTCCC |
| HPRT | TGGAAAAACGAAATACAAACGATGAA | ATTCGCCCCAAAAGGACTGATGATG |
| SMARCA2 | GGAACAGAATGAACTGAAAGGTC | AAAAAACCGGCTTCTATATGAC |
| TBP | GCTTCTGCGGTAATATGGA | CTTCTGCGGTAATATGGA |
| HHIP | AGAATGCAAAAATGTAGCCAG | TCCGTAGAAGAATACGTCCGTC |

**ChIP analysis**

For ChIP analysis, proteins and DNA were fixed with a final concentration of 10% formaldehyde in the cell medium for 10 min and quenched with glycine. The cells were gently washed twice with PBS containing 1× Complete (Roche) protease inhibitor and then removed by scraping the cells in cold PBS with 1× Complete and transferring to a clean tube. The cells were pelleted by centrifugation, suspended in ChIP lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% Nonidet P-40, 20 mM Tris, pH 8.1, 1× Complete) for 20 min followed by centrifugation to pellet the nuclei. The nuclei were suspended in nuclear lysis buffer (150 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, 1× Complete). Chromatin was sheared with sonication for 32 cycles (30-s on/off cycles) in a Diogenade Biorupter 300. After the shearing check, the samples were centrifuged to remove the debris, and the supernatant was transferred to a clean tube. The samples were then equally aliquoted with 2 E 6 cells per IP, diluted 1:10 with dilution buffer (0.01% SDS, 1% Triton, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167 mM NaCl, 1× Complete), and incubated with 2 μg of IgG control or a specific antibody overnight with slow rotation at 4 °C. Dynabeads Protein A/G (Thermo Fisher) were washed one time with dilution buffer, and 30 μl were added to each sample and allowed react for 4 h with slow rotation at 4 °C. The beads were gently washed three times with dilution buffer, and the DNA was eluted with 100 μl of elution Buffer (26.4 mM Tris-HCl, 0.01% NaOH, 0.01% HCl, 200 mM NaCl, 1% SDS) plus 1 μl of proteinase K and incubation for 2 h at 65 °C and 95 °C for 10 min. DNA was purified using a DNA purification spin column kit (Qiagen). Extracted DNA was diluted 1:20 with water and PCR-amplified with validated ChIP primers and SYBR Green detection; Gal4 UAS domain forward, GCATGCGATATTGCGCGAC, and reverse, AGATGAGACGACACTCCGAGTTG; and HHIP putative enhancer (Chr 4: 145206838–145207375) forward, TTGGATAAGGACTGATGAGATCC and reverse, ATGTGCCATAGACAGACCC.

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**J. Biol. Chem. (2020) 295(26) 8725–8735 8733**
GLI1 and SMARCA2 regulate chromatin accessibility

Immunoprecipitations

Cells were harvested by trypsinization, counted with a hemocytometer, pelleted by centrifugation, and washed twice with cold PBS containing 1× complete (Roche) protease inhibitor. The cell pellet was frozen on dry ice then transferred to a −20 °C freezer until further preparation. The cell pellet was thawed and suspended in buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, pH adjusted to 7.5 at 4 °C and 1× complete) for 20 min with occasional mixing. The cell lysate was centrifuged for 10 min at 13,000 × g and 4 °C, and the supernatant was transferred to a clean tube. 50-μl aliquots were saved as input, the lysate protein was quantitated with the BCA method, and 2E6 cells were aliquoted for each IP/co-IP. The lysate was diluted 1:2 with dilution buffer (50 mM Tris, pH 7.5, 0.5% Nonidet P-40, pH adjusted to 7.5 at 4 °C with 1× complete). 1 μg of antibody was bound to the protein over-night at 4 °C, and then 30 μl of Dynabeads added and incubated for 4 h at 4 °C. The beads were washed twice for 10 min with 200 μl of lysis buffer diluted 1:2 with the dilution buffer with 1× complete (Roche) protease inhibitor. 10 μl of PAGE loading buffer was used to suspend the beads and transfer everything to a clean tube. The diluted lysis buffer was completely removed, and 15 μl of PBS with 1× complete and 15 μl of PAGE loading buffer was added. The analysis continued with PAGE and WB.

Data Availability

All the described data are contained within this article. The ATAC and RNA-seq data have been deposited in the Gene Expression Omnibus under accession numbers GSE143681 and GSE143684.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: MNase, micrococcal nuclease; ATAC-seq, transposase-accessible chromatin with sequencing; TAD, transactivation domain; DBD, DNA-binding domain; aa, amino acid(s); WB, Western blotting; BS, binding site; IP, immunoprecipitation; FDR, false discovery rate; TSS, transcription start site; HA, hemagglutinin; PCAF, P300/CBP-associated factor; DE, differential expression.

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GLI1 and SMARCA2 regulate chromatin accessibility

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