Hedgehog pathway-regulated gene networks in cerebellum development and tumorigenesis

Eunice Y. Lee[a], Hongkai Ji[b], Zhengqing Ouyang[c], Baiyu Zhou[d], Wenxiu Ma[e], Steven A. Vokes[f], Andrew P. McMahon[g], Wing H. Wong[d], and Matthew P. Scott[a,1]

[a]Departments of Developmental Biology, Genetics, and Bioengineering, Howard Hughes Medical Institute, and Departments of [c]Biology, [d]Statistics and Health Research and Policy, and [e]Computer Science, Stanford University, Stanford, CA 94305; [b]Department of Biostatistics, Johns Hopkins University, Baltimore, MD, 21205; [f]Section of Molecular Cell and Development Biology, Institute for Cellular and Molecular Biology, University of Texas, Austin, TX 78712; and [g]Department of Molecular and Cellular Biology and Harvard Stem Cell Institute, Harvard University, Cambridge, MA 02138

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Many genes initially identified for their roles in cell fate determination or signaling during development can have a significant impact on tumorigenesis. In the developing cerebellum, Sonic hedgehog (Shh) stimulates the proliferation of granule neuron precursor cells (GNPs) by activating the Gli transcription factors. Inappropriate activation of Shh target genes results in unrestrained cell division and eventually medulloblastoma, the most common pediatric brain malignancy. We find dramatic differences in the gene networks that are directly driven by the Gli1 transcription factor in GNPs and medulloblastoma. Gli1 binding location analysis revealed hundreds of genomic loci bound by Gli1 in normal and cancer cells. Only one third of the genes bound by Gli1 in GNPs were also bound in tumor cells. Correlation with gene expression levels indicated that 116 genes were preferentially transcribed in tumors, whereas 132 genes were target genes in both GNPs and medulloblastoma. Quantitative PCR and in situ hybridization for some putative target genes support their direct regulation by Gli. The results indicate that transformation of normal GNPs into deadly tumor cells is accompanied by a distinct set of Gli-regulated genes and may provide candidates for targeted therapies.

Hedgehog | Gli | transcription | cerebellum | medulloblastoma

The Hedgehog (Hh) pathway, discovered in *Drosophila*, has emerged as a major developmental pathway in many organisms, from insects to mammals (1, 2). Sonic hedgehog (Shh) is critical for patterning, proliferation, and differentiation in a variety of tissues. In the cerebellum, Shh is a potent mitogen for granule neuron precursor cells (GNPs) (3, 4). Shh is emitted from Purkinje cells and binds to and inactivates Patched1 (Ptch1), a receptor on GNPs, unleashing the Smoothened transmembrane protein to cause the production of activator forms of the Gli transcription factors (2). The Gli proteins are modified in structure and location to differentially repress or activate target genes, which in turn alter cell properties.

The Gli proteins have distinct and overlapping roles. Loss of Gli2 or Gli3 causes severe defects and premature lethality in mice. Gli1 is not essential for development (5, 6). Although Gli2 is the initial activator during development of several tissues, including the cerebellum, Gli1 amplifies the Gli2-driven response to Shh (6–8). Moreover, Gli1, whose transcription is directly induced by Shh signaling, completely compensates for Gli2 loss when replaced into the *Gli2* locus (9, 10). Therefore, Gli1 and Gli2 are functionally redundant and presumably act upon many of the same targets.

Activation of Hh target genes is associated with cancers including medulloblastoma (MB), a malignant tumor in the cerebellum. Shh signaling was first implicated in MB by mutations in the human *PATCHED1* gene that cause Gorlin's syndrome, an inherited disorder associated with multiple clinical features (11, 12). An inactivating mutation in *Ptch1* also predisposes mice to develop MB (13). Reduced Ptch1 function results in excess expression of Gli-regulated target genes, allowing some GNPs to continue cell division.

Few direct Gli target genes have been identified in the cerebellum, including *Ptch1*, *Ptch2*, *Gli1*, and *N-Myc* (14–17). These genes influence murine MB development (18–21), so at least part of the Shh-stimulated transcriptional program in GNPs is active and functional during carcinogenesis. Genome-wide expression profiling has been widely used to understand the transcriptional effects of Shh signaling, but it does not distinguish direct and indirect effects. Many questions remain regarding the repertoire and regulation of Hh target genes and the extent of their overlap in different tissues and cancers.

An approach using epitope-tagged Gli protein enabled the identification of Hh regulatory circuitry governing neural specification (22) and limb patterning (23). To identify genes directly regulated by Gli1 in GNPs and primary MBs, we used this strategy in combination with gene expression profiling. Our study uncovered target genes previously linked to Shh signaling and, most importantly, previously unknown regulators likely to play important roles in the cerebellum. Identifying transcriptional targets that differ between normal and diseased states may provide ways to specifically target tumors.

Results

Epitope-Tagged Gli1 Does Not Alter Cerebellum Development or Tumorigenesis. To identify Shh target genes during cerebellar development and tumorigenesis, we determined the genomic locations where the Gli1 transcription factor binds in GNPs and MBs. Because of the lack of antibodies that efficiently precipitate endogenous Gli proteins, we used antibodies against the FLAG epitope to isolate Gli1-bound chromatin from mice carrying a conditional FLAG-tagged *Gli1* transgene, $Rosa^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$ (22). Gli1-FLAG was produced in GNPs by breeding $Rosa^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$ animals to mice expressing *Cre* under the control of the *Math1* promoter (24). This promoter is active in dividing GNPs and in MBs. The *Gli1-FLAG* transgene contains an internal ribosome entry site upstream of *Yfp*, causing independent translation of YFP to mark cells that produce Gli1-FLAG. YFP was not observed in the brains of postnatal day 8 (P8) $Rosa^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$ animals without *Cre* (Fig. 1*A*). In $Rosa^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$;*Math1-Cre*/+ littermates, YFP was in the cerebellum. Gli1-FLAG protein was present in cerebellum extracts from $Rosa^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$;*Math1-Cre*/+ animals, but not from control animals (Fig. 1*B*).

Expression of FLAG-tagged Gli1 neither detectably affected cerebellum development nor induced tumor formation. Ectopic

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[1]To whom correspondence should be addressed. E-mail: mscott@stanford.edu.

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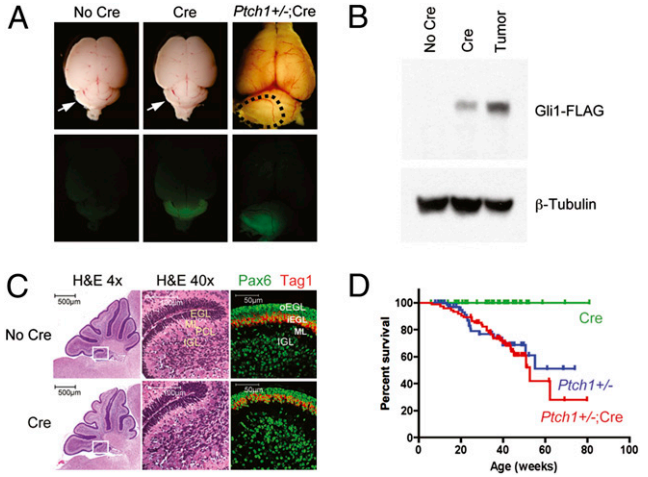

Fig. 1. Expression of FLAG-tagged Gli1 in the cerebellum. (*A*) Dorsal views of brains from P8 *Rosa*$^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$ (No Cre), P8 *Rosa*$^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$;*Math1-Cre*/+ (Cre), and adult *Rosa*$^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$;*Math1-Cre*/+;*Ptch1*$^{+/-}$ (*Ptch1*$^{+/-}$;Cre) mice. Arrows indicate cerebellum. *Ptch1*$^{+/-}$;Cre MB is demarcated by dotted line. YFP (*Lower*) is restricted to P8 Cre cerebellum and tumor. [Objective: ×1 (P8) and ×0.8 (adult).] (*B*) Gli1-FLAG is present in Cre cerebellum and tumor extracts. (*C*) H&E-stained P8 cerebellum sections at ×4 (*Left*) and ×40 (*Center*). Merge of Pax6 and Tag1 immunostaining (*Right*) reveals similar morphologies of No Cre and Cre cerebellums. Pax6 is in GNPs and granule neurons. Tag1 is in GNPs in the inner external granule layer (iEGL). oEGL, outer EGL; ML, molecular layer; PCL, Purkinje cell layer; IGL, internal granule layer. (*D*) Kaplan-Meier curves show survival rates for *Rosa*$^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$;*Math1-Cre*/+ (Cre, green, n = 53), *Rosa*$^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$;*Ptch1*$^{+/-}$ (*Ptch1*$^{+/-}$, blue, n = 67), and *Rosa*$^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$;*Math1-Cre*/+; *Ptch1*$^{+/-}$ (*Ptch1*$^{+/-}$;Cre, red, n = 74) mice.

Gli1 expression can cause abnormal neural tube development (25) and tumors in the skin (26). Histological and immunohistochemical stainings of cerebellar sections from mice with or without Cre did not reveal any morphological differences (Fig. 1*C*). The lack of phenotypic effects suggests that an inconsequential amount of additional Gli1 was made. To rule out low-level recombination in GNPs producing Gli1-FLAG, isolated GNP populations were analyzed by flow cytometry. Eighty-nine percent of GNPs purified from Gli1-FLAG animals were YFP positive, compared with 0.3% of control GNPs lacking Cre (Fig. S1*A*). Thus, the majority of *Rosa*$^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$;*Math1-Cre*/+ GNPs underwent recombination by P8. We also compared gene expression profiles from GNPs producing Gli1-FLAG and GNPs without Cre. Expression levels of known Shh transcriptional targets *Ptch1*, *Ptch2*, and *N-Myc* were similar for both genotypes (Fig. S1*B*). Elevated *Gli1* transcripts in *Gli1-FLAG*-expressing cells were due to expression of *Gli1-FLAG*, not heightened activation of Shh targets.

For tumor studies, we crossed FLAG-tagged Gli1-expressing animals with *Ptch1*$^{+/-}$ mice (13). In *Rosa*$^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$;*Math1-Cre*/+;*Ptch1*$^{+/-}$ mice that developed MB, YFP and Gli1-FLAG proteins were detected in tumors (Fig. 1 *A* and *B*). The survival rate and frequency of MB were similar in *Rosa*$^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$;*Math1-Cre*/+;*Ptch1*$^{+/-}$ animals and *Rosa*$^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$;*Ptch1*$^{+/-}$ mice (Fig. 1*D*), indicating that tagged Gli1 production does not modulate tumor formation. Thus, Gli1-FLAG did not significantly impact gene expression, GNP development, or oncogenesis in the cerebellum.

Many Gli1-Bound Regions Are Not Shared by GNPs and MBs. We detected 3,514 and 4,486 Gli1-binding regions in GNPs and MB [false-discovery rate (FDR) = 0.05], respectively, by chromatin immunoprecipitation and microarray analysis (ChIP-chip) using Affymetrix promoter tiling arrays. In Gli1-bound regions, the Gli binding motif was enriched >2-fold compared with genomic control regions (Fig. S2 *A* and *B*).

To verify the ChIP-chip results, we performed quantitative PCR (qPCR) for a subset of Gli1-bound sequences using unamplified ChIP samples. Gli1 binding to 71 regions from GNPs and 81 regions from MB was measured in two to four biological replicates (Fig. S2 *C* and *D*). A region was deemed a true positive if it showed ≥3.5-fold enrichment of Gli1 binding in more than half the samples. With a local FDR cutoff of 50%, we defined the top 510 regions in GNPs and the top 1,060 regions in MBs as high-confidence Gli1-bound regions (HCGRs) (Table S1). The high degree of phylogenetic sequence conservation of the HCGRs, compared with noncoding and random genome sequences, suggests that these regions are biologically significant (Fig. S2 *E* and *F*).

Oncogenic activation of Shh signaling in GNP-restricted progenitor cells gives rise to MBs (27, 28). The developing cerebellum and MB express many of the same genes (14, 16). Thus, we expected substantial concordance among the genomic regions occupied by Gli1 in GNPs and MB. Previously identified enhancers of Hh target genes *Ptch1*, *Ptch2*, and *Gli1* were bound by Gli1 in both cell types, but remarkably, comparison of all HCGRs identified only 168 common regions. We considered the possibility that only the strongest ChIP signals are indicative of important targets. Strikingly, 42% of the top 50 regions in GNPs and MB were in common (Fig. S2*G*); substantial differences were detected in even the highest-ranked regions. The variation in Gli1 chromatin occupancy suggests major changes in direct Gli1 targets during oncogenic transformation of GNPs.

Binding Motifs Within Gli1-Bound Regions. Gli1 could act alone or in partnership with other transcription factors. Using an unbiased approach, HCGRs were analyzed for enriched sequence motifs de novo. Two significant motifs emerged (Table 1). The first motif resembled the known Gli binding site (22, 29, 30) and was in approximately half of the regions in both cell types (Table 1).

The second enriched motif contains an E-box element (CANNTG) that is typically recognized by basic helix–loop–helix (bHLH) proteins (31). This motif was present in 26% of GNP regions and in 46% of MB regions (Table 1). In MBs, occurrence of an E-box motif is slightly higher in regions without Gli motifs. In GNPs, the appearance of an E-box motif is more frequent when a Gli motif is present. Upon comparison with all HCGRs, the linkage of E-box and Gli motifs was significantly more frequent than in control sequences (Table S2). Thus, Gli1 may cooperate with an E-box binding protein to regulate gene expression.

Gli1-Bound Regions Can Activate Transcription in Response to Gli1. A set of HCGRs was tested to determine whether Gli1 acts through the putative enhancers to control gene expression. We assayed 29 DNA fragments: 24 had at least one Gli consensus motif, and 5 did not (Table S3). Gli1 bound to all of the chosen regions in MB cells and to eight of the regions in GNPs. Two previously characterized Shh-responsive promoter fragments from *Ptch1* and *Ptch2*, also identified in our study, were included as positive controls.

Each Gli1-bound region was placed upstream of a Firefly luciferase gene and assessed for transcription activation potential in response to transfected Gli1 in NIH 3T3 fibroblasts. Of the 27 putative new enhancers, 5 responded to Gli1 (Table S3). One enhancer (near *Cltb*) stimulated robust reporter activity far greater than observed for the *Ptch* enhancers. Two enhancers (near *Gpr153* and *Ncor2*) yielded levels similar to the *Ptch* enhancers, and two enhancers (near *Sox18* and *Ncor2*) induced low reporter activity. The five sequences without a Gli motif were unresponsive. A Gli motif is not sufficient to guarantee a response, because 17 regions with a Gli motif were unresponsive. The lack of responsiveness may be due to the need for additional sequences, weak Gli1 binding in NIH 3T3s, lack of a cofactor, or positioning requirements with respect to a promoter.

To address the possibility that some candidate enhancers are regulated in a cell type–dependent manner, reporter assays were performed in PZP53 cells, a cell line derived from a murine *Ptch1*$^{+/-}$;

Table 1. Enriched de novo motifs in Gli1-bound regions

Motif	Parameter	GNPs: High-confidence regions (total 510)	GNPs: Regions with Gli motif (total 273)	GNPs: Regions without Gli motif (total 237)	Medulloblastoma: High-confidence regions (total 1,060)	Medulloblastoma: Regions with Gli motif (total 519)	Medulloblastoma: Regions without Gli motif (total 541)
 "Gli"	No. with motif	273	—	—	519	—	—
	Enrichment*	2.61	—	—	3.35	—	—
	95% CI	2.24–3.10	—	—	2.93–3.90	—	—
 "E-Box"	No. with motif	133	89	44	491	243	248
	Enrichment*	0.97	1.12	0.72	1.81	1.48	1.87
	95% CI	0.84–1.12	0.93–1.35	0.58–0.90	1.65–2.01	1.30–1.69	1.61–2.16

CI, confidence interval.

*Enrichments were calculated as ratios: percentage of regions containing the indicated motif(s) vs. percentage of control regions with the same motif(s). Enrichment ratios were computed 1,000 times, each time with a different set of control regions. Control regions were randomly selected such that the distance between the control region and the closest transcription start site (TSS) has the same probability distribution as the distance between the TileMap ranked region and the TSS.

p53$^{-/-}$ MB (32). These cells are not responsive to Shh, so assays were performed with transfected Gli1. Five enhancers responded to Gli1: three (near *Sox18*, *Cltb*, and *Ncor2*) that responded in fibroblasts, and two (near *Boc* and *Tle1*) that were activated only in PZP53 cells (Table S3). Thus, some cis-regulatory sequences respond to Gli1 in two cell types and others in one. In both cell lines, the ability of an element to drive reporter expression was highly correlated with the TileMap ranking (correlation coefficient, 0.53–0.58).

Gli sites identified from our motif analysis were mutated and tested for reporter gene activation. Mutation of Gli motifs significantly reduced or abolished reporter expression in all nine cases examined (Table S4). Five regions contained two Gli motifs. For the region near *Sox18*, each Gli motif was necessary for gene regulation. Both Gli motifs in the *Ptch1*-associated region were important, but to different extents. One was absolutely required to respond to Gli1, whereas the other allowed <20% residual activity. Only one of two Gli motifs within the enhancers near *Ptch2* and *Boc* was required for Gli1 responsiveness. Thus, all Gli binding motifs are not equal. The relative importance of each site was proportional to how closely the site matched the consensus motif, TGGGTGGTC, so differential activities of the Gli motifs may reflect their affinity for Gli1. There was no correlation between how well the enhancer matched the Gli consensus and the level of reporter activity.

E-box motifs were mutated in three enhancers that responded to Gli1. Mutation of the E-box sites in two enhancers (near *Boc* and *Cltb*) had little or no effect on reporter induction in NIH 3T3 and PZP53 cells (Table S4). Mutation of one E-box site near *Tle1*, but not the other, significantly reduced Gli1 responsiveness. Which bHLH protein, if any, binds to the E-box site remains unknown.

Combining Gli1 Binding with Gene Expression to Identify Cell Type-Specific Target Genes. The dramatic alteration in chromatin binding sites may indicate changes in target gene regulation or may reflect transcription-independent chromosomal associations of Gli1. To distinguish these possibilities, three gene expression analyses were performed: (*i*) genes expressed in Gli1-FLAG GNPs, (*ii*) genes expressed in MBs, and (*iii*) genes induced in MBs vs. *Ptch1*$^{+/-}$ GNPs. We focused on genes potentially induced by Gli1, because Gli1 acts solely as a transcriptional activator (33). A total of 9,260 genes were expressed in Gli1-FLAG GNPs, and 9,185 genes were expressed in MBs; 8,691 were in common. The substantial overlap is consistent with the cellular origin of MB (27, 28).

To identify Gli1-regulated genes, we assigned each HCGR to the closest gene. This yielded 474 GNP target genes and 845 MB target genes (Fig. 2, tier I). Combining expression data with Gli1 binding, 420 genes in GNPs and 633 genes in MB are putative candidates for direct Gli regulation ($P = 3.6 \times 10^{-38}$ for GNPs; $P = 2.4 \times 10^{-15}$ for MB; Fig. 2, tier II; Table S5). One hundred thirty-two genes were common to GNPs and MBs (Fig. 2, tier III) and included well-established Gli1 target genes. Gene ontology (GO) terms associated with these genes included *cell proliferation* and *Smoothened signaling* (Table S6). The most enriched protein domain is the bHLH dimerization region, which may bind the E-box site prevalent in Gli1-bound regions. Four transcriptionally active genes were associated with Gli1 binding exclusively in GNPs, and 46 genes were active and bound solely in MBs (Fig. 2, tier V; Table S5).

Additional MB-specific targets had nearby Gli1 binding and higher expression in MBs. We compared gene expression in MBs with that in *Ptch1*$^{+/-}$ GNPs, the cell type of origin for these tumors, and the results were integrated with our binding and expression findings (Fig. 2, tier VI). A fold change ≥1.7 and FDR ≤0.05 were deemed appropriate cutoffs because *N-myc*, a direct Gli1 target, was 1.74 times higher in tumors than in GNPs. Among the 449 genes that were expressed in both cell types that had nearby Gli1 binding only in MB cells, 70 genes had increased expression in MB (Fig. 2, tier VI; Table S5). These genes are likely to be MB-specific Gli1 targets. The 280 putative GNP-specific Gli1 targets (Fig. 2, tier IV) could not be further assessed, because Shh pathway activation in Gli1-FLAG GNPs was similar to that in control cells. Yet, it is clear that the transcriptional network directed by Gli1 is considerably altered during the transition from normal development to cancer, because MBs have at least 116 distinctive Gli1 target genes (Fig. 2, tiers V and VI).

Distinguishing Direct Gli1 Target Genes. Direct Gli target genes should increase their expression in response to Shh despite a block in protein synthesis. We cultured WT GNPs for 6 hours in the presence of Shh, with or without the protein synthesis inhibitor cycloheximide (CHX). Quantitative RT-PCR was used to measure relative transcript levels of 27 candidate genes. *Gli1*, itself a direct Shh target, served as a positive control. Of the targets examined, transcripts of seven genes (*Boc*, *Cdk6*, *Foxo6*, *Gpr153*, *Ncor2*, *Olig2*, and *Gli1*) were induced by Shh approximately 2-fold more than in untreated GNPs (Fig. 3*A*). When cells were also treated with CHX, expression levels of four genes were still induced (*Foxo6*, *Gpr153*, *Ncor2*, and *Gli1*), consistent with them being direct Gli targets (Fig. 3*B*). It was not possible to test transcriptional effects of Shh in tumor cells, because available MB cell lines are unresponsive to Shh treatment.

To further test putative Gli targets, we performed qPCR and in situ hybridization. *Gpr153* seems to be a direct Gli target in GNPs and tumors. Consistent with *Gli1* expression pattern, *Gpr153* was expressed in GNPs at P2 and P6, and in MBs (Fig. 3 *C* and *D*).

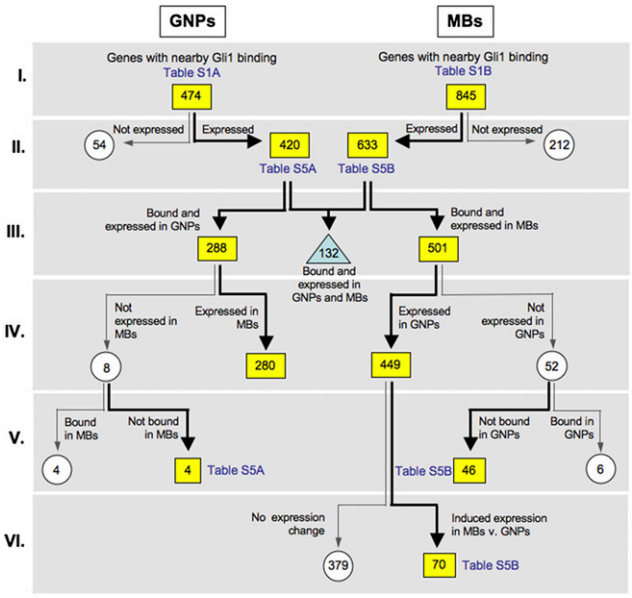

Fig. 2. Flowchart of genes classified by Gli1 chromatin localization and gene expression analyses. Putative Gli1 target genes with nearby Gli1-binding regions in GNPs and MBs are shown in tier I. Correlations with gene expression are in tier II. Tier III represents tier II genes that are in common (blue triangle) or distinct between GNPs and MBs. Nonoverlapping tier III genes were assessed by expression levels in the other cell type, shown in tier IV. The genes expressed and bound in one cell type, and not expressed in the other (tier IV) were subdivided according to Gli1 binding properties in the other cell type (tier V). Genes expressed in both cell types and bound by Gli1 exclusively in MBs (tier IV) were categorized by relative expression levels (MBs vs. *Ptch1*$^{+/-}$ GNPs) in tier VI. The yellow-boxed numbers are highlighted in the text. Supplemental tables for gene lists are denoted.

qPCR showed that three of four GNP-specific genes (*Celsr1*, *Rbpms*, and *D4Bwg0951e*; Fig. 2, tier V) were expressed at significantly higher levels in purified GNPs than in MBs (Fig. 3*C*). Their mRNA levels were too low to be detectable by in situ hybridization.

Three genes (*Akna*, *Nptx1*, and *Olig2*; Fig. 2, tier V) were more highly expressed in MBs than in GNPs (Fig. 3*C*). The in situ data for five MB-specific targets show expression in tumor cells, not GNPs (Fig. 3*D*). *Akna* was not detectably expressed in developing cerebellum but had low expression in tumors. *Cltb* was highly expressed in Purkinje cells at P6, not GNPs, and was strongly expressed in MB, as was *Nptx1* (Fig. 3*D* and Fig. S1*C*). *Olig1* and *Olig2* were expressed in subsets of cells within the tumor. *Olig* expression may be a result of intermingling oligodendrocytes, but Olig2 has been linked to MB formation (28). These results confirm that we have successfully identified Gli-regulated target genes.

Discussion

Identifying Direct Targets from Gli1 Occupancy and Expression Data. We identified a total of 1,151 genes with nearby Gli1 binding in GNPs and MBs out of >25,000 promoters interrogated by ChIP-chip. Our detection of previously characterized enhancers of known Gli targets, including *Ptch1*, *Ptch2*, *Gli1*, and *Rab34* (22, 33, 34), adds confidence that newly identified putative enhancers are meaningful. The majority of the genes identified as potential Gli targets (921 of 1,151) were detectably expressed in GNPs or MBs. Three of 26 candidate genes were confirmed as direct targets because they were induced by Shh in the absence of new protein synthesis. Some CHX-sensitive targets may also be direct Gli target genes, because their expression could require both Gli and another factor induced by Shh. In addition, all MB-specific targets may not be validated in GNPs, so the actual success rate may be higher. It is also possible that the few residual granule neurons within tumors may mask normal targets as MB targets. Despite these possibilities, we have verified direct Gli regulation of tested genes, and the results imply that at least 100 genes are direct Gli targets.

Many locations where Gli1 bound do not clearly contribute to transcriptional events. Some do not contain a conserved Gli binding motif, and some do not reside near transcriptionally active genes. These binding events may be unrelated to transcriptional regulation of protein-coding genes. Transcription factor binding regions with no clear involvement in gene expression have been identified in *Drosophila* (35) and for Gli3 in mice (23). These studies found that the majority of transcription factor binding regions are nonfunctional. This may be the case for Gli1 as well. In some cases, Gli1 may act at long distances, precluding easy linkage to regulated genes. Long-range enhancers have been identified as far as 1 Mb (for *Shh*) (36). Long-range elements have also been described for Gli3 (23). If Gli1 can exert its action over long distances, then some HCGRs could be incorrectly assigned to the nearest gene.

Mechanisms Underlying Cell Type-Specific Target Genes. If altered Gli1 targeting is unique to cancer cells, Gli1 should occupy similar chromosome locations in various normal tissues. A previous study identified 25 Gli1-bound genomic sites in neural progenitor cells (22), 17 of which were represented on the microarrays used in our study. In GNPs, Gli1 bound to 5 of the 17 regions, all of which were associated with Hh targets common to many tissues (*Ptch1*, *Ptch2*, *Gli1*, and *Rab34*). Ten additional regions bound by Gli1 in GNPs were not observed in neural progenitor cells, although the sequences were represented on both arrays. Thus, Gli1-controlled transcriptional networks are cell type-specific, irrespective of cancer cells constituting an aberrant cell type.

Combinatorial or competitive control of cis-regulatory elements by multiple Gli proteins may contribute to differences in Gli1 localization between GNPs and MBs. Gli1 and Gli2 mainly function as transcriptional activators, and Gli3 acts primarily as a transcriptional repressor. These proteins can bind to the same DNA motif, reflecting their highly similar zinc finger DNA binding domains (29, 30). Thus, Gli2 or Gli3 could displace Gli1 binding under certain conditions. In the developing limb, repressor Gli3 is bound to regions normally recognized by Gli1 in neural progenitor cells (23). Our results show that 4 and 46 potential Gli1-regulated target genes in GNPs or MBs, respectively, (Fig. 2, tier V) had exclusive expression and Gli1 binding. Although Gli1 and Gli3 may not share all of the same targets, it is possible that at these loci, Gli3 may prevent Gli1-mediated activation of a gene in a cell type–dependent manner.

Cofactors could influence in vivo binding properties of Gli1. The E-box motif we described may be related to one detected in a subset of Gli3-bound regions in limb buds (23). bHLH proteins, which recognize E-box motifs, have critical roles in developmental processes and cancer (31). There is no precedent for direct cooperation between Gli and bHLH proteins, but Olig2, a bHLH protein, interacts with zinc finger protein Zfp488 during oligodendrocyte differentiation (37). Several genes encoding bHLH proteins are expressed in GNPs and MBs, and some were among the direct Gli1 target genes. Thus, Gli1 and one or more bHLH proteins may cooperate in the cerebellum and other Hh-responsive tissues, although the requirement of the E-box motif on Gli-regulated gene expression remains unclear.

Roles of Target Genes in Normal Development and Tumorigenesis. Functional classification of Gli target genes highlights the diversity in signaling pathways and gene functions regulated by Gli1 in each cell type. *Notch signaling* and *neurogenesis* were processes exclusive to the MB targets (Table S6). Notch signaling has been implicated in murine and human MB (38, 39), yet how Shh and Notch signaling are connected is unclear. Gli may induce Notch-related genes. With regard to neuron development, Gli1 may trigger inappropriate expression of certain GNP genes in tumor cells. Alternatively, genes involved in axon guidance and neurite

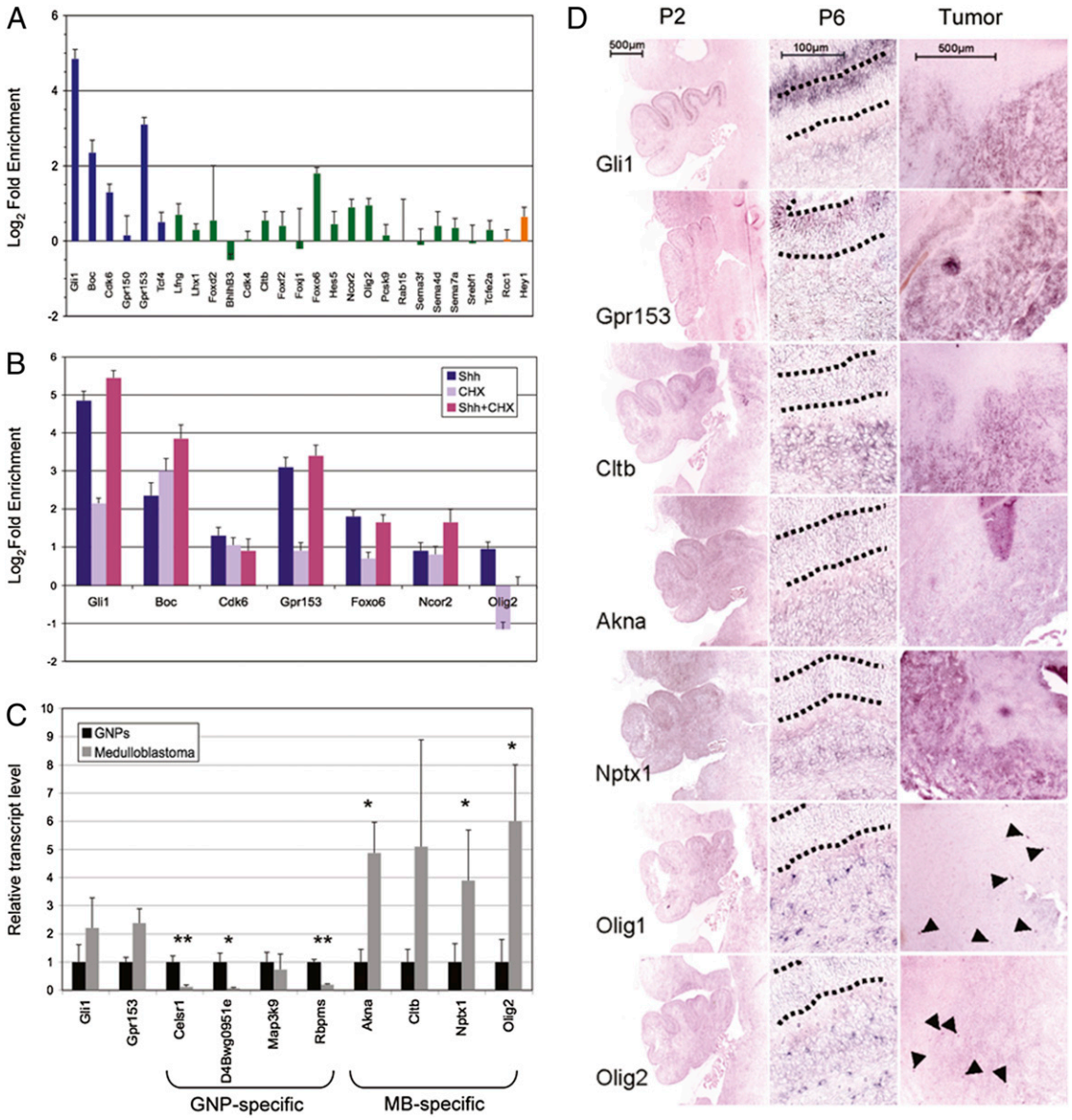

Fig. 3. Validation of direct Gli1-regulated genes. RNA quantification (±SE) of (*A*) putative Gli1-regulated genes in WT GNPs cultured with Shh for 6 h; (*B*) putative Gli1 targets in WT GNPs treated 6 h with Shh and/or CHX, a protein synthesis inhibitor; and (*C*) putative GNP-specific and MB-specific targets in freshly isolated GNP and MB samples. In *A*, expression levels are shown for genes with nearby Gli1 binding in GNPs and MBs (blue), in only tumors (green), and in only GNPs (orange). In *C*, two-tailed equal variance *t* test determined significance. $*P < 0.05$; $**P < 0.006$. All values are normalized to *Gapdh* and compared with untreated GNPs. (*D*) In situ hybridization of Gli1 target genes in P2 (×4) and P6 (×40) midline sagittal cerebellum sections and in MB tumors (×10). Dotted lines outline external granule layer composed of GNPs (P6). *Gli1* is strongly expressed in GNPs and in tumor cells, as is *Gpr153*. *Cltb*, *Akna*, *Nptx1*, *Olig1*, and *Olig2* are not detected in GNPs, but are present in tumors. Arrows indicate positively stained cells.

growth have been implicated in angiogenesis during tumor formation (40). Gli1 may regulate these targets in MB cells for this reason.

Our study identified three unique Gli targets in the cerebellum: *Gpr153*, *Foxo6*, and *Ncor2*. Biological functions of Gpr153 and FoxO6 remain unexplored, but Ncor2 (or SMRT) is a transcriptional corepressor linked to Notch and retinoic acid signaling (41). *Ncor2* has not previously been implicated in MB, but is critical for maintaining the undifferentiated state of cortical progenitor cells in vitro (41). Thus, Ncor2 may maintain the progenitor and proliferation properties of GNPs and in the same way may contribute to MB.

The distinct sets of potential target genes identified in GNPs and MBs compel us to reassess the transcriptional events that regulate GNP development and MB formation. How Gli directs expression of different subsets of genes in a context-dependent manner is a key issue to understand how Shh signaling drives different cell states.

Materials and Methods

Mouse Strains and Cell Isolation. *Rosa*$^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$ mice (22) were crossed to *Math1Cre/+* mice (24). P8 cerebella were harvested into PBS and assessed for YFP. Cerebellar GNPs were purified by percoll fractionation (3, 42) and used for ChIP or qPCR. *Rosa*$^{Gli1\text{-}FLAG/Gli1\text{-}FLAG}$;*Math1-Cre/+* mice were bred to *Ptch1*$^{+/-}$ (13) mice. MBs were isolated at 10–30 weeks of age under a dissection stereomicroscope. MB samples for RNA isolation were frozen in liquid nitrogen and those for ChIP were digested in trypsin-EDTA (Invitrogen). After 15 min, Neurobasal (Invitrogen) with 10% FBS (Thermo Scientific) was added. Single cell suspensions were obtained by pipetting. Kaplan-Meier survival curves were generated with Prism 4.0c software.

ChIP-chip. ChIP and ligation-mediated PCR were performed as previously described (22) with few modifications. Briefly, 2–7 × 10^6 cells were used per immunoprecipitation with anti-FLAG M2 antibody (Sigma). Chromatin was fragmented with a Branson 450 Sonifier (two pulses at 45%, eight at 50%). After linker ligation, each sample was divided for two PCR reactions. Samples were then pooled, purified, and tested by qPCR. Samples (5–7.5 μg) were hybridized to Mouse Promoter 1.0R tiling arrays (Affymetrix) according to the manufacturer's instructions. Three biological replicates were analyzed per cell type. ChIP-chip data were analyzed using the TileMap moving average method (43). Further details are provided in *SI Materials and Methods*. ChIP-chip data are accessible through Gene Expression Omnibus (GEO) Series accession no. GSE17682 in the National Center for Biotechnology Information (NCBI)'s GEO (44).

Gene Expression Profiling. Total RNA was isolated with TRIzol reagent (Invitrogen) and analyzed on an Agilent bioanalyzer 2100. One microgram of RNA was used per sample and hybridized to Mouse Exon 1.0ST arrays according to Affymetrix instructions. Expression indices were calculated using the GeneBASE program (45) from background-corrected probe signals. To determine detectable expression of a gene, the combined *Z* score statistic of each transcript cluster from core probes was calculated. The *Z* test was used to obtain *P* values. A *P* value <0.05 indicated detectable expression.

Differential gene expression in MBs vs. GNPs was identified using Significance Analysis of Microarrays (46). The DAVID database (National Institute of Allergy and Infectious Diseases) designated genes based on GO terms and Interpro domains (47). Expression data are available in NCBI's GEO (44), accession no. GSE17702 (part of SuperSeries GSE17719). ChIP-chip and gene expression correlation is described in *SI Materials and Methods*.

Reporter Assays and Biochemistry. For luciferase assays, 25 ng pCIG-Gli1 or pCAGG-RFP, 80 ng reporter vector (enhancer cloned into pGL4.23; Promega), and 20 ng Renilla luciferase vector were transfected into NIH 3T3 fibroblasts or PZP53 cells with Fugene 6 (Roche). Mutations were introduced using QuikChangeII XL (Stratagene).

For Shh and CHX experiments, WT P8 GNPs were isolated and cultured on matrigel- (BD Biosciences) coated plates and treated with 10 μg/mL Shh-N and/or 10 μg/mL CHX (Sigma). For target validation, freshly isolated GNPs and MBs were used. Three or four biological replicates were assayed. RNA was isolated with TRIzol (Invitrogen). cDNA was made with SuperScriptIII (Invitrogen). SYBR green master mix (Qiagen) and 50 ng cDNA or 1/60 ChIP DNA was used per qPCR reaction in iCycler real-time PCR system (Bio-Rad). Primers to assess Gli1-bound regions and gene expression are listed in Table S7.

For Western blot, anti-FLAG (Sigma) and anti-β-tubulin (Covance) antibodies were used. For immunostaining, anti-Pax6 (Covance) and anti-Tag1 (DSHB) were used. In situ hybridization is described in *SI Materials and Methods*.

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