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An Integrated Approach Identifies *Nhlh1* and *Insm1* as Sonic Hedgehog–regulated Genes in Developing Cerebellum and Medulloblastoma[1,2]

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Abstract

Medulloblastoma (MB) is the most common malignant brain tumor of childhood arising from deregulated cerebellar development. Sonic Hedgehog (Shh) pathway plays a critical role in cerebellar development and its aberrant expression has been identified in MB. Gene expression profiling of cerebella from 1- to 14-day-old mice unveiled a cluster of genes whose expression correlates with the levels of Hedgehog (HH) activity. From this cluster, we identified *Insm1* and *Nhlh1/NSCL1* as novel HH targets induced by Shh treatment in cultured cerebellar granule cell progenitors. *Nhlh1* promoter was found to be bound and activated by Gli1 transcription factor. Remarkably, the expression of these genes is also upregulated in mouse and human HH–dependent MBs, suggesting that they may be either a part of the HH-induced tumorigenic process or a specific trait of HH-dependent tumor cells.

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Introduction

Medulloblastoma (MB) is the most common malignant pediatric brain tumor, whose prognosis has not improved significantly in the last two decades, despite multimodal therapy (surgery, radiation, and chemotherapy), thus justifying the continuous effort in better characterizing the molecular mechanisms involved in tumor initiation and progression [1,2].

MB is commonly recognized to originate from cerebellar granule or other precursors that fail to differentiate and keep proliferating [3]. Indeed, the transcriptional pattern of MB is similar to that of the developing mouse cerebellum (5–10 days postpartum), thus supporting the concept that MB is formed from immature cerebellar precursor cells that retain most of the undifferentiated characteristics [4].

Cerebellar development in mammals largely occurs in the early postnatal period. In mice, proliferation and expansion of the most external layer of the cerebellum, i.e., the external granule layer (EGL), start at birth and peak by 8 to 10 days postpartum (P) [5]. Subsequently, EGL cell proliferation starts exhausting, most of the cells migrate through the molecular and Purkinje cell layers, reach their final resting state, and extend dendrites in the internal granule layer until EGL disappears and differentiation is completed [6].

A major regulator of this process is the Hedgehog (HH) pathway [7]. Sonic Hedgehog (Shh) is a glycoprotein secreted by Purkinje cells, which binds to the transmembrane receptor Patched (Ptc) on

Abbreviations: EGL, external granule layer; EMSA, electrophoretic mobility shift assay; GCP, granule cell precursor; GST, glutathione-*S*-transferase; HH, Hedgehog; MB, medulloblastoma; Ptc, Patched; QPCR, quantitative polymerase chain reaction; Shh, Sonic Hedgehog

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[2]This article refers to supplementary material, which is designated by Table W1 and is available online at www.neoplasia.com.
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granule cell precursors (GCPs) and, through the activation of downstream transcription factors of the Gli family, promotes proliferation of the EGL progenitors [8]. Interestingly, at the stage of maximal GCP proliferation, HH is maximally activated, as documented by the increase of its target genes such as N-*myc*, Cyclin D2 (*Ccnd2*), and *PDGFRα* [4,9].

Aberrant activation of the Shh pathway has been linked to MB formation [10,11]. Germline mutation of Ptch1 (the Shh receptor which acts as a repressor in the absence of the ligand) is responsible for the Gorlin syndrome that exhibits high MB incidence [12]. Mutations of the HH pathway negative regulators (e.g., Ptch1, SuFu, and Ren/KCTD11) were also reported in sporadic MB (reviewed in the works of Wetmore _Hlt183838176[[10], Ferretti et al. [11], and Di Marcotullio et al. [13]), as well as in the upregulation of Shh target genes, such as *Gli1* [10]. Similarly, different mouse models that exhibit increased rate of MB underscore the direct relationship between Shh/Ptc1 signaling and MB [10]. Shh transcriptional targets involved in the mitogenic response of mice GCPs (e.g., *Ccnd2* and N-*myc*) were confirmed to be implicated in MB development [14,15], further highlighting the role of altered Shh signaling in MB.

To further characterize the contribution of the HH pathway in cerebellar development and MB and to search for novel Shh targets potentially useful for patient stratification and/or molecular therapies we: 1) used gene expression profiling of mouse cerebella at different time points during postnatal development (P1 through P14) to identify subsets of genes specifically upregulated in the time window in which HH pathway is strongly activated; 2) selected several of these genes and verified if they are induced in cultured cerebellar GCPs treated with Shh; and 3) analyzed mouse and human MB samples for the expression levels of these genes.

Through this approach, we have identified *Insm1* and *Nhlh1/NSCL1* as new HH target genes that are overexpressed in MB. Through the identification of functional Gli binding sites on the promoters of mouse and human *Nhlh1*, we also highlighted a direct regulation of this gene by Gli transcription factors.

Materials and Methods

Tissue Samples, Cell Cultures, and Treatments

Surgical specimens of human MBs were collected with the approval of the Institutional Review Board. Control human cerebella RNA were from Biocat (Heidelberg, Germany) and Ambion (Foster City, CA).

Mouse cerebella and MB specimens were obtained from wild-type and *Ptc1*$^{+/-}$ CD1 mice [16]. Animal handling was according to the guidelines of Sapienza University of Rome. Mouse GCP cultures were prepared cultured and treated as previously described [17]. Recombinant Shh N-terminal peptide was from R&D Systems (Minneapolis, MN).

HEK293T cells were maintained in DMEM containing 10% FBS, glutamine, and antibiotics.

Microarrays and Real-Time Quantitative Polymerase Chain Reaction Analysis

Total RNA from P1 to P14 mice cerebella (six mice at each time point) was used. RNA extraction, cDNA synthesis, cRNA labeling, hybridization, and scanning were performed according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). For each of the duplicate microarray hybridization, RNA from three mice of the same time point was pooled. GeneChip Murine Genome U74Av2 Set microarrays (Affymetrix) were used in all microarray experiments. Data analysis was performed using Time-Course Experiment AMDA version 2.1.0 (Milan, Italy).

Real-time quantitative polymerase chain reaction (QPCR) was performed as previously described [13] using the ABI Prism 7900HT System (Applied Biosystems, Monza, Italy), with Assay-on-Demand reagents (Applied Biosystems). mRNA quantification was expressed in arbitrary units as sample/calibrator ratio or sample/mean of controls ratio. All values were normalized to endogenous controls: glyceraldehyde-3-phosphate dehydrogenase, β-actin, and hypoxanthine–guanine phosphoribosyltransferase.

Constructs and Luciferase Assays

pGL4 and pRLTK reporters were from Promega (Madison, IL). pGL4-5xmutGliBS, pGL4-5xmGliBS, pGL4-3xhGliBS, and pGL4-5xhGliBS were generated by ligation of synthetic oligos (sequences are shown in Figure 4*A*) into pGL4. pCMVGliHA was previously described [13]. Flag-tagged Gli1 was cloned into pCDNA (Invitrogen, Carlsbad, CA) by PCR using the *Not* I site. pGEX–GliZF was cloned by PCR and fused in-frame in pGEX4t3 (Amersham, Milan, Italy). All constructs were verified by sequencing.

For luciferase experiments, HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen, Milan, Italy) according to the manufacturer's instructions. About 24 to 48 hours later, luciferase assay was performed using a dual-luciferase kit (Promega, Milan, Italy).

Electrophoretic Mobility Shift Assay

The double-stranded DNA fragments corresponding to the mouse and human promoter regions (Figure 3*A*) were PCR-amplified and purified by electrophoresis and gel extraction kit (Qiagen, Milan, Italy). The canonical Gli binding sequence (Figure 3*A*) was produced by annealing complementary oligos. Fragments were ^{32}P-labeled, purified through G30 columns (Amersham Pharmacia, Milan, Italy), and used for electrophoretic mobility shift assay (EMSA).

Nuclear extracts were prepared from 293T cells transfected with pCDNAFlagGli1 or an empty vector. Glutathione *S*-transferase (GST) fusion proteins were produced in BL21 cells following isopropyl-β-D-1-thiogalactopyranoside (IPTG) induction, cell lysis, and binding to glutathione–agarose beads (Amersham Pharmacia) according to manufacturer's instructions.

EMSA was performed according to standard procedures. Binding reactions contained 20,000 counts per minute ^{32}P-labeled probe and 15 μg nuclear extract (or the indicated amount of GST fusion protein) in binding buffer. In some experiments, 2 μg of rabbit anti-GLI1 (H-300; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti–FLAG-M2 (Sigma) antibodies were added to the sample. Competition experiments were performed by adding 50× or 100× molar excess of cold oligonucleotides. Complexes were resolved on a non-5% PAGE, dried, and exposed for autoradiography.

Western Blot Analysis

Tissue samples were lysed in standard radioimmunoprecipitation assay buffer plus protease inhibitors (Roche, Mannheim, Germany). Lysates were separated on SDS-PAGE and immunoblotted using standard procedures. Anti-NSCL1 (AB5698; Chemicon, Milan, Italy), anti–β-actin, and HRP-conjugated secondary antisera (Santa Cruz Biotechnology, Heidelberg, Germany) were used, followed by enhanced chemiluminescence (ECL; Amersham).

Results

Gene Expression Profiling of Developing Mouse Cerebellum

Quantitative analysis of Gli1 mRNA expression levels throughout mouse postnatal development shows a peak between P7 and P10 (Figure 1*A*), indicating the highest HH pathway activation in this timeframe.

Because putative HH target genes are expected to be regulated in an overlapping period, we have analyzed gene expression in mouse cerebella during the first 2 weeks of development, by means of the GeneChip Murine microarray (Affymetrix). RNA samples obtained from six mice cerebella collected at each time point (P1, P2, P7, and P14) were used. Analysis of about 12,000 genes present in the microarrays allowed us to identify several clusters of genes with diverse trends of expression during cerebellar development (Figure 1*B* and not shown). We focus here on the analysis of a subset of genes specifically upregulated between days 2 and 14, with a peak around day 7, and reduced expression afterwards (Figure 1*B*). As expected, some of the genes that belong to this cluster are known targets of HH pathway (e.g., *Ccnd2*, *PDGFRα*,

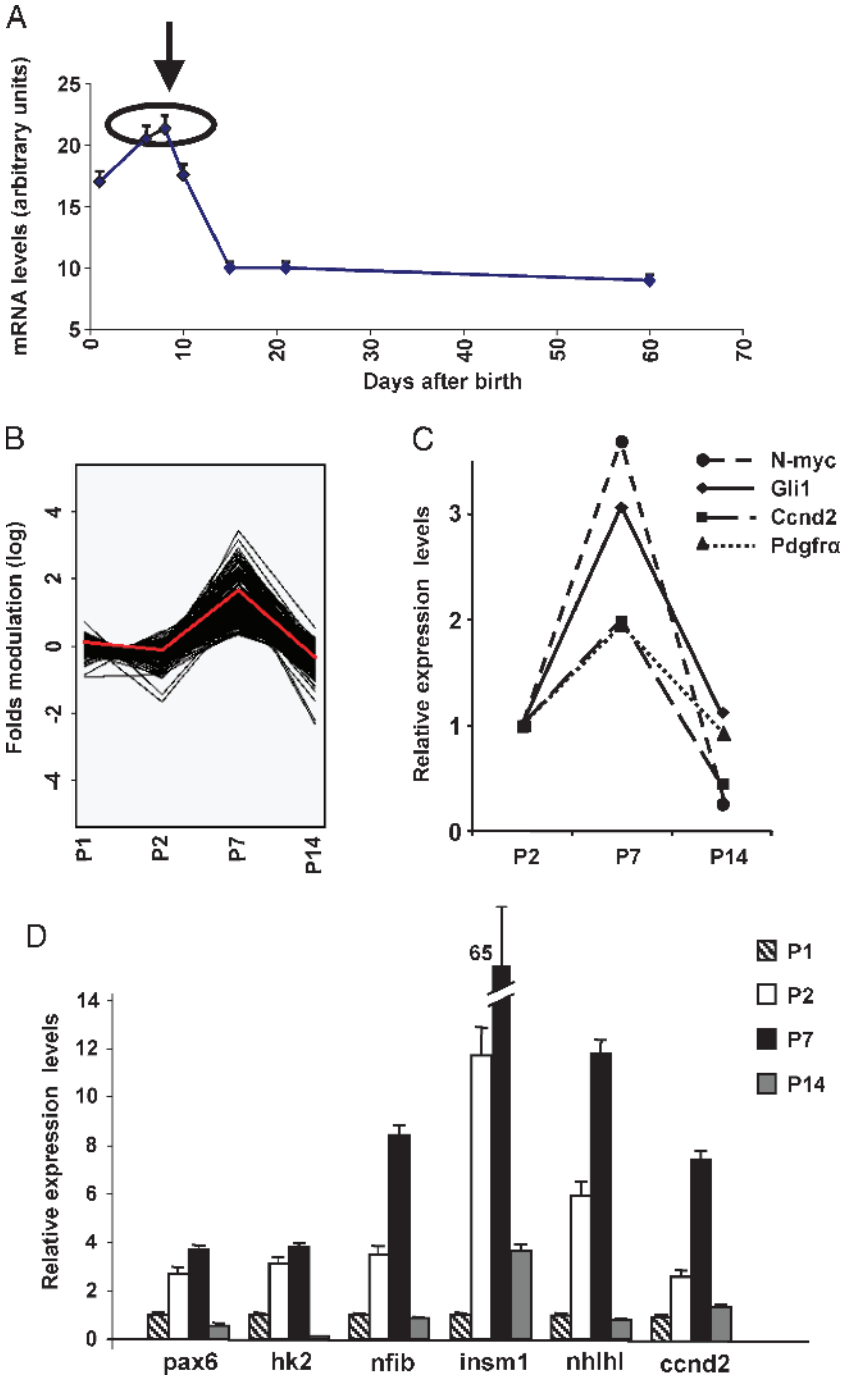

Figure 1. Identification of genes whose expression mirrors Hedgehog activity during mouse cerebellar development. (A) Modulation of *Gli1* expression in mouse cerebella during development. (B) Cluster of genes with peak of expression at P7, from microarrays data. (C) Expression levels of Shh target genes during cerebellar development (data from microarrays). (D) QPCR analysis on the same mRNA samples used for microarray hybridization (triplicate experiments).

N-*myc*, and *Gli1* itself [18,19]) (Figure 1*C*). Table W1 shows a selection of the most strongly modulated genes belonging to this cluster.

The developmentally regulated expression of several genes (including HH targets such as *Ccnd2*) was further confirmed by QPCR analysis on the same mRNA samples used in the microarrays (representative experiments in Figure 1*D* and not shown). Between the most strongly upregulated genes were the transcription factors neural basic helix–loop–helix 1 (*Nhlh1*) [20] and insulinoma-associated 1/IA-1 (*Insm1*) [21] (Figure 1*D*).

Gene Expression Analysis in Shh-Treated Cultured Cerebellar GCPs

The main cerebellar HH target cell is the GCP, which also represents the most abundant cerebellar cell population. Therefore, following the hypothesis that several genes upregulated at P7 during cerebellar development should also be HH targets, we measured expression levels in primary GCPs (from P4 mice) cultured in the presence or in the absence of recombinant Shh-N protein. A significant increase in *Gli1* and *Ccnd2* mRNA levels confirmed the HH activation in Shh-treated GCPs (Figure 2, *A* and *D*). *Nhlh1* levels in control GCPs decrease significantly throughout the time of culture (from 24 to 96 hours) (Figure 2*B*), suggesting that *Nhlh1* expression is sustained by *in vivo* signals that are progressively lost during the *in vitro* culture. One of these *in vivo* signals is represented by Shh: in fact, the addition of Shh antagonizes silencing of the *Nhlh1* gene in cultured GCPs, suggesting that Shh activates *Nhlh1* transcription (Figure 2*B*). Moreover, *Insm1* expression levels significantly increase upon Shh treatment (Figure 2*C*), whereas the levels of several additional genes examined, including *Pax6* (Figure 2*E*), *Nfib*, and *HK2* (data not shown), were not significantly modulated.

These data suggest that *Nhlh1* and *Insm1* are targets of the HH signaling pathway.

Nhlh1 *Is a Direct Target of* Gli1 *Transcription Factor*

We next analyzed *Insm1* and *Nhlh1* promoter regions for the presence of putative Gli-responsive sites. Whereas analysis of the *Insm1* promoter did not reveal potential Gli binding sites (not shown), analysis of the promoter region of mouse *Nhlh1* allowed the identification

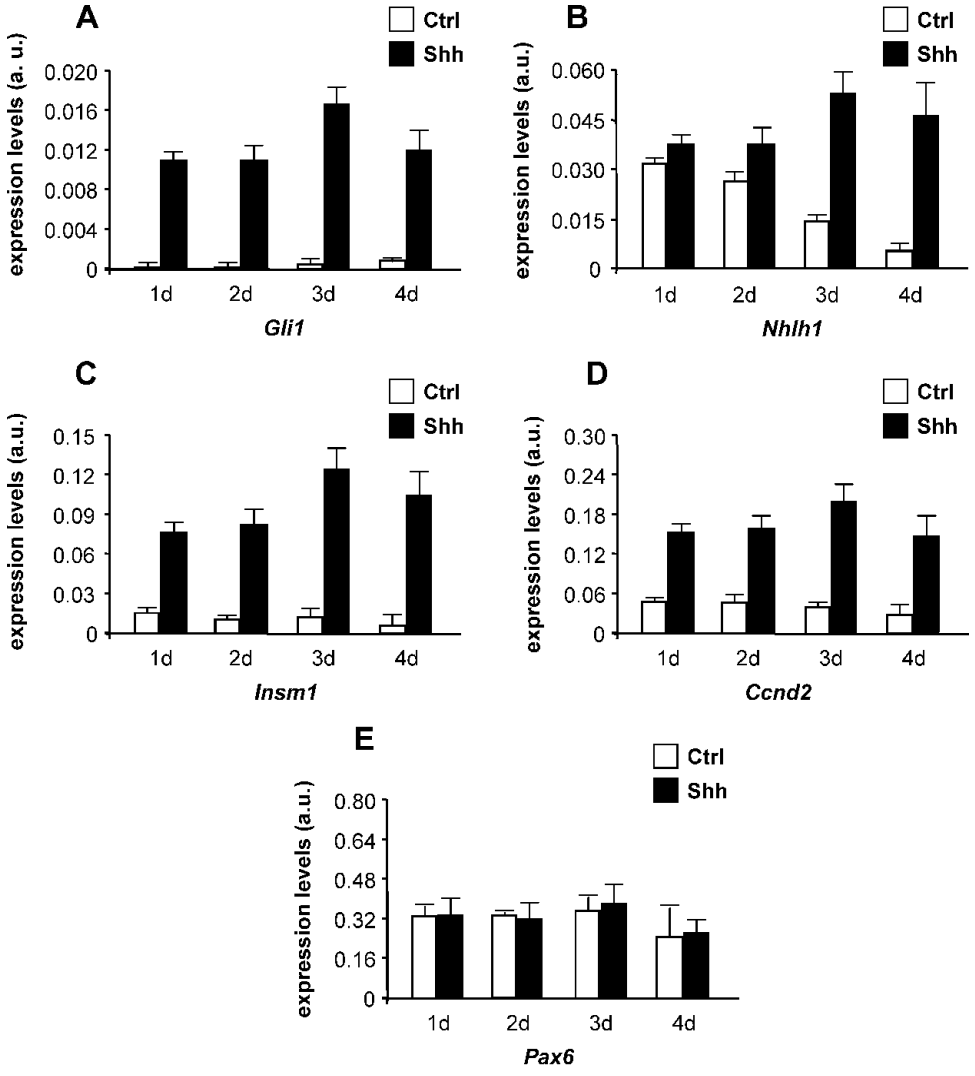

Figure 2. Shh induces transcription of *Nhlh1* and *Insm1*. (A–D) Primary GCPs from P4 mice cerebella were cultured with or without Shh for 4 days (1d–4d). Relative levels of (A) *Gli1*, (B) *Nhlh*, (C) *Insm*, (D) *Ccnd2*, and (E) *Pax6* at the different time points are represented (averages from triplicate experiments; *a.u.*, arbitrary units).

of a potential Gli binding site, located approximately 700-bp (−702/−690) upstream of the transcriptional start site, which was partially conserved also in the upstream region of the human sequence (−695/−685; see Figures 3*A* and 4*A*).

To verify whether *Nhlh1* promoter was actually bound by Gli1, we performed EMSA using nuclear extracts from Flag-tagged Gli1-transfected 293T cells and a DNA probe containing the mouse promoter fragment spanning between −736 and −615 bp (shown in Figure 3*A*). Figure 3*B* shows that Gli1 binds the promoter fragment (mNhlh) and this binding is competed by either a 50× cold probe excess or addition of anti-Gli1 or anti-Flag antibodies. The presence of a Gli-promoter complex was confirmed by the efficient

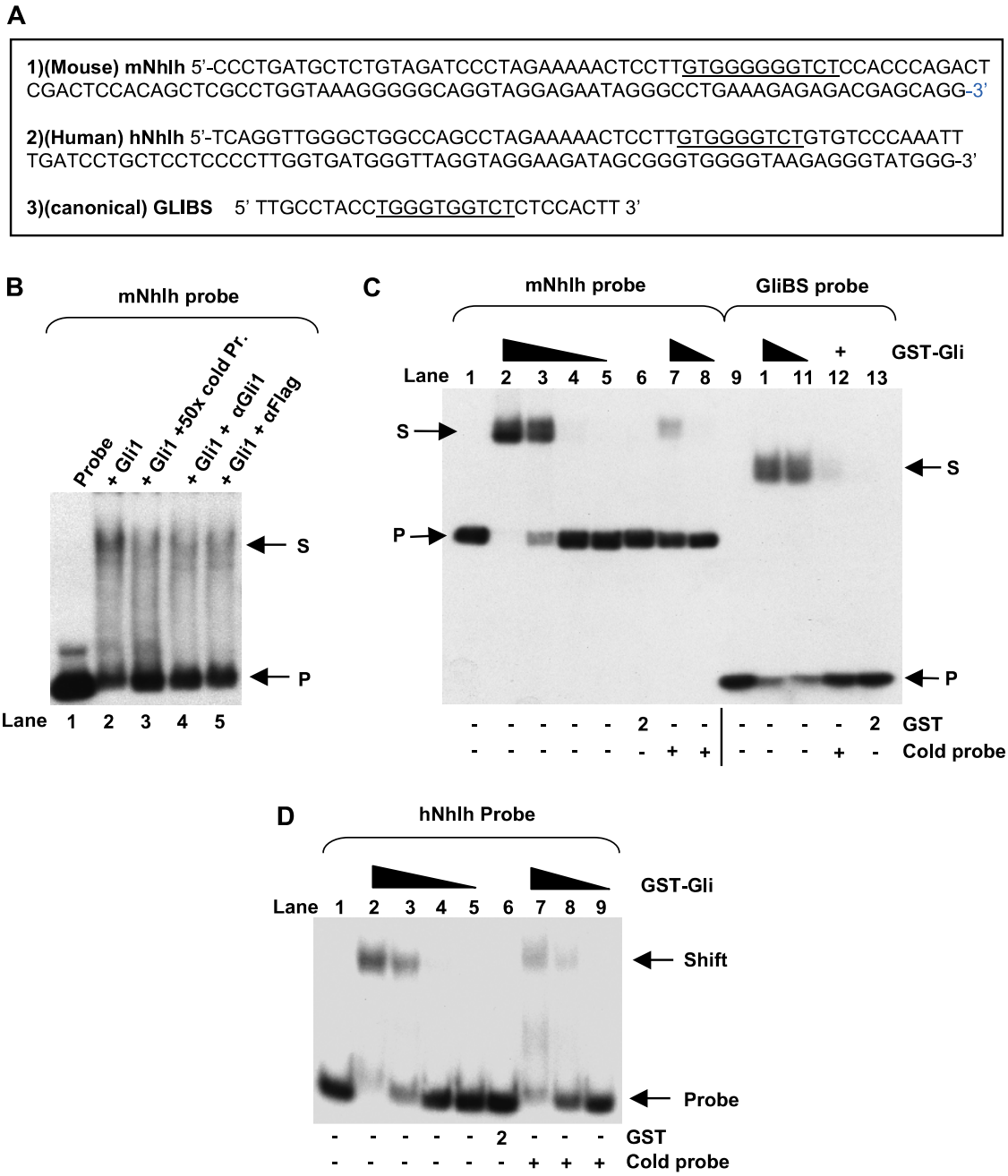

Figure 3. Gli transcription factor binds *in vitro* to the putative Gli-binding sequences on the *Nhlh1* promoters. (A) Mouse (1) and human (2) *Nhlh1* promoter sequence containing the Gli-responsive regions (underlined). Sequence (3) is a canonical GliBS–containing fragment [22]. Double-stranded oligonucleotides containing these sequences were used in the following EMSA. (B) EMSA of the mouse *Nhlh* probe (P) was performed using lysates from cells transfected with Gli1–Flag–expressing vector. The shifted complex (S) is competed with a 50× excess of cold probe or by incubation with antibodies α-Gli1 or α-flag. (C) EMSA using recombinant GST–Gli (Gli1 zinc-finger fragment: aa 242–424). Lanes 1 to 8: GST–Gli binds with good affinity and in linear scale to the labeled *mNhlh* probe (lanes 2–5: used 2–0.2 μg of GST–Gli), whereas GST does not bind (lane 6: used 2 μg). Binding can be competed by 100× unlabeled probe (lanes 7 and 8). Lanes 9 to 13: binding of GST–Gli to a canonical GliBS (lane 9: free probe; lanes 10 and 11: binding to GST–Gli (2 and 1 μg); lane 12: 100× cold competition; lane 13: binding to GST alone). (D) Same experiment as in (C) lanes 1 to 8, but using the *hNhlh* probe.

and quantitative binding of recombinant GST-fused Gli1 zinc-finger to the mNhlh probe (which was competed by a 100× excess cold probe), whereas the corresponding GST alone did not bind at all (Figure 3*C*). As a positive control, binding of a canonical GliBS-containing fragment [22] to the same GST–Gli1 protein indicates a comparable affinity of the two using the human *Nhlh1* promoter probe (*hNhlh*: position −730/−607), whose complex with GST–Gli1 was competed by an excess of cold probe (Figure 3*D*), whereas GST alone failed to bind DNA.

To verify whether the interaction between Gli1 and these Gli-binding sites was able to trigger transcription, luciferase reporter constructs containing the Gli-binding sites from either mouse (*mNhlh*-GliBS) or human (*hNhlh*-GliBS) *Nhlh1* promoters (Figure 4*A*) were used in luciferase assays. *mNhlh*-GliBS reporter increased luciferase levels following cotransfection with increasing amounts of Gli1 vector, whereas activity of mutant GliBS (mutGliBS, unable to bind Gli1) or the empty reporter was not modulated (Figure 4*B*). Similarly, human GliBS reporters significantly enhanced luciferase transcription in response to increasing amounts of transfected Gli1 (Figure 4*C*).

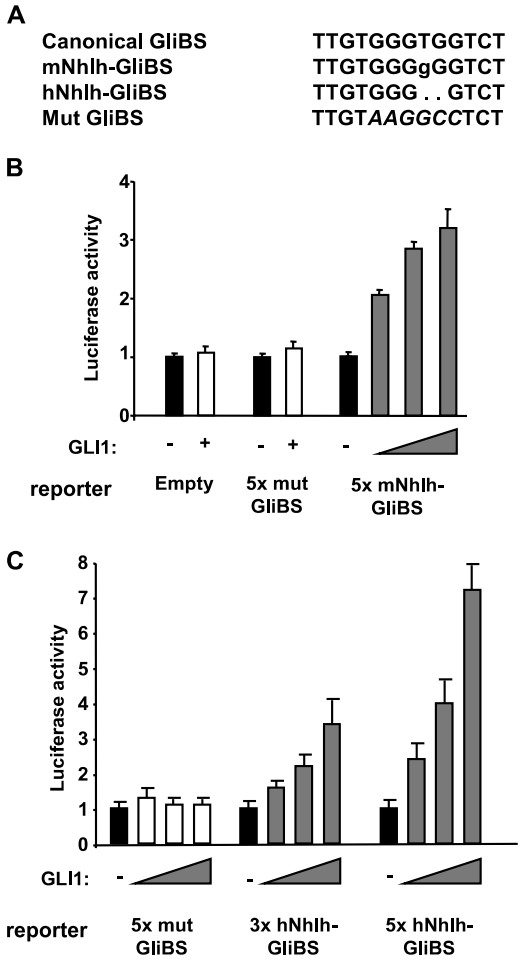

Figure 4. Gli-Binding sites on *Nhlh* are transcriptionally activated by Gli1. (A) Comparison of a canonical Gli–binding site [22] and Gli-binding sites on mouse and human *Nhlh* promoters (*mNhlh*-GliBS and *hNhlh*-GliBS, respectively). At the bottom, a mutated binding site (mutGliBS) was used as a negative control. (B) Activation of a *mNhlh*-GliBS luciferase reporter (five copies of the sequence), following cotransfection with increasing amounts (5, 20, and 50 ng) of Gli1-expressing vector. Empty reporter and mut-GliBS (five copies) are cotransfected as a control with 50 ng of Gli1 plasmid. (C) Same assay as in (B), but using *hNhlh*-GliBS (three or five copies) and mutGliBS as reporters (average ± SD from at least three experiments).

These results indicate that *Nhlh* is a direct target of HH signaling pathway and is transcriptionally activated by Gli1.

Expression Analysis of Insm1 and Nhlh1 in MB Samples

To test whether Insm1 and Nhlh1, modulated during cerebellar development, could also be relevant in MB, we evaluated their expression in tumors obtained from *Patched1*$^{+/-}$ mice (*Ptc*$^{+/-}$) in which GCPs develop HH-dependent MB due to the loss of the receptor Ptc, which leads to the constitutive activity of the pathway [16,23].

Confirming HH pathway activation in these tumors, all MB from *Ptc*$^{+/-}$ animals exhibited high levels of HH activity compared to adult and P7 cerebella as documented by *Gli1* (Figure 5*D*) and *Ccnd2* mRNA levels (Figure 5*C*). Remarkably, we observed a dramatic increase of both *Insm1* and *Nhlh1* mRNA levels in all MBs (Figure 5, *A*–*C*), suggesting that HH-triggered expression of these genes is conserved during cerebellar tumorigenesis *in vivo*. The high *Nhlh1* levels expressed in mouse MB samples were also mirrored by increased protein levels when compared either to normal adult control (Figure 5*E*) or to primary granule cells obtained from P4 mice cerebella (Figure 5*F*). Interestingly, relative levels of Nhlh1 protein samples from P2 and P7 cerebella controls were higher than in adult, in keeping with mRNA expression levels shown in Figure 1*D*.

Next, we investigated whether the deregulated expression of *Insm1* and *Nhlh1* observed in mouse HH–dependent MB was also relevant in the multifactorial carcinogenesis of sporadic human MBs. To this purpose, we analyzed a set of 16 human sporadic MBs (Figure 6). *Insm1* was expressed at higher levels in tumors compared to either adult ($P < .05$) or fetal cerebella (Figure 6*A*). Likewise, *Nhlh1* was overexpressed in all tumor samples compared to adult ($P < .005$) and fetal cerebella (Figure 6*B*).

Most MBs respond to the HH inhibitor cyclopamine with a block of proliferation, implying a certain degree of basal HH activity [19]. However, we recently reported that human sporadic MBs might be subclassified as low- or high-*Gli1*–expressing, indicating a different level of HH activation [17].

We have therefore subdivided our series of MBs in two subsets: MBs with *Gli1* levels at least two standard deviations above the mean value of the adult cerebella (Gli^{high} subset) and MBs with *Gli1* levels within or below the range of normal cerebella (Gli^{low}) (Figure 6*C*). We then analyzed *Insm1* and *Nhlh1* levels in these two subsets. Although *Insm1* levels in the Gli^{high} subset were more significantly increased ($P < .02$) compared to adult cerebella, we could not correlate *Gli1* and *Insm1* expression levels on the whole MB population (Figure 6*D*), because of the high *Insm1* levels found frequently in Gli^{low} tumors (Figure 6, *A* and *C*), suggesting a more complex regulation of *Insm1* in MB, in addition to HH control. Instead we found a significant correlation between *Gli1* and *Nhlh1* levels ($P < .02$) in the two MB subset (Figure 6*E*), consistent with a direct role of HH in the regulation of *Nhlh1* expression.

Discussion

Our work identifies the developmentally regulated genes *Insm1* and *Nhlh1* as novel targets of HH activity. Most relevantly, the expression

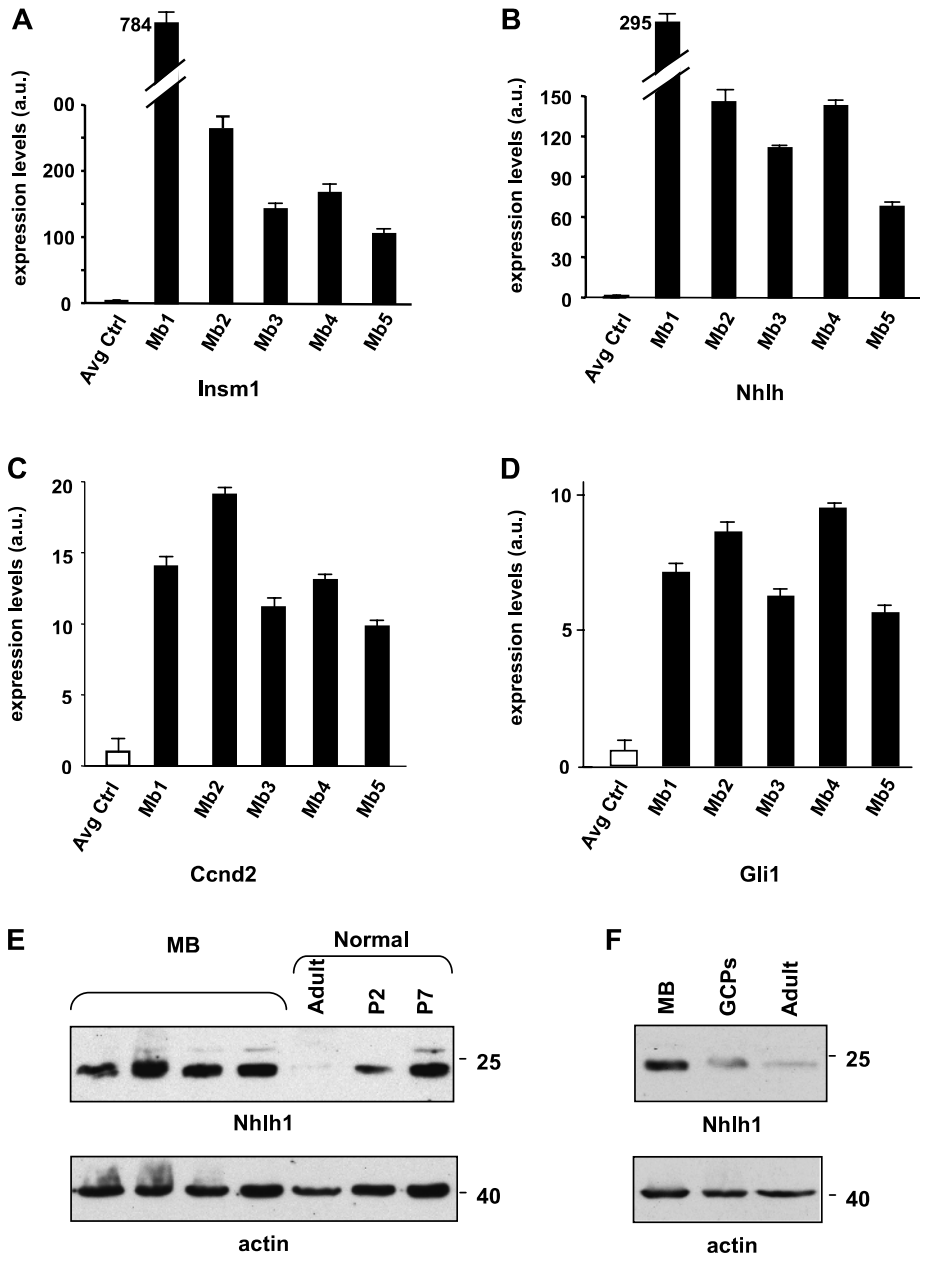

Figure 5. Increased *Insm1* and *Nhlh1* expression in Hedgehog-dependent mouse MB. (A–D) *Insm1*, *Nhlh1*, *Ccnd2*, and *Gli1* mRNA expression in different MB samples compared to P7 and adult cerebellum controls (average value from six cerebella). Quantitations have been performed in triplicate experiments (*a.u.*, arbitrary units). (E–F) Nhlh1 protein levels in MBs and mouse cerebella correlate with mRNA expression. Western blot showing Nhlh1 protein levels in: (E) four mouse MB and normal adult, P2 and P7 cerebella; (F) mouse MB, GCPs and adult. β-Actin is shown as a loading control.

of these genes is upregulated in both mouse and human MBs, in which HH pathway is activated, suggesting that they may be either a part of the HH-induced tumorigenic process or a specific trait of HH-dependent tumor cells.

Insm1 encodes a zinc-finger DNA-binding protein and exhibits a restricted expression pattern, including fetal brain [21,24], pancreas [25], and neuroendocrine tumors [21,26].

We report here on the activation of *Insm1* expression by HH signaling. The *Insm1* promoter does not present Gli-binding sites, suggesting that the mechanisms of activation by Shh may be due either 1) to the presence of Gli-binding sites in other *cis*-regulatory regions of the gene or 2) to indirect regulation, through some yet unidentified intermediate transcription factors. To date, the transcriptional activation of *Insm1* in neuroendocrine tissues and tumors has been attributed to the formation of basic helix–loop–helix (bHLH) protein heterodimers, NeuroD/E47 and neurogenin3/E47 [27], on the E-box of *Insm1* promoter [24,28]. Whether these factors are involved in the regulation of *Insm1* by HH remains to be elucidated.

Interestingly, the link between HH pathway and *Insm1* we have shown in brain tumors is consistent with the *Insm1* expression in the majority of small cell lung cancers [26], which also exhibit a high frequency of HH pathway activation [29]. Furthermore, increased *Insm1* had been previously detected in desmoplastic MB, together with N-*myc*, *Ptc*, and *Gli1*, which well fits with increased HH activation in this subclass of MB [30].

The biologic functions of Insm1 are not well defined: it has been shown that Insm1 acts as a transcriptional repressor on *NeuroD* and on *Insm1* itself, generating an autoregolatory loop [31]. It was also shown that Insm1 is required for the differentiation of endocrine pancreas [25,28], whereas its role in cerebellar or neuronal development has not been identified yet [25]. Likewise, the role of Insm1 in MB needs to be clarified. The regulatory loop between Insm1 and NeuroD, a factor important in the differentiation and survival of postmitotic cerebellar granule cells, may explain, in part, its role in neuronal development and tumorigenesis [32].

As for the second HH target *Nhlh1*, this gene was originally cloned because of its homology within the bHLH motif to the oncogenic hematopoietic transcription factor SCL/Tal1 [20]. Nhlh1 belongs to class II of the bHLH protein family of transcriptional regulators [27]. Class II bHLHs exhibit tissue-restricted expression and some of them (e.g., neurogenin, NeuroD, and Math1) are expressed in neuronal cells and are involved in the specification of neuronal lineages. Nhlh1 acts as a transcriptional repressor through dimerization with E47 (a class I bHLH) and interacts with the Lim-only family members LMO4 and LMO2 that modulate its activity [33].

So far, analysis of *Nhlh1* transcripts [20,34] or use of lacZ reporter [35] indicate that *Nhlh1* expression is confined to the nervous system. *Nhlh1* expression has been observed in both the embryonic and postnatal developing cerebellum. Between E14.5 and E18.5, *Nhlh1* expression is restricted to migrating GCP of the cerebellar epithelium, which originates from the rhombic lip to form the EGL [35,36]. Postnatally, *Nhlh1* is expressed in the premigratory zone of the EGL with maximal expression between P7 and P10 [37], in agreement with our findings (Figure 1*D*). At P10, *Nhlh1* transient expression is also detected in the newly formed internal granule layer [35],

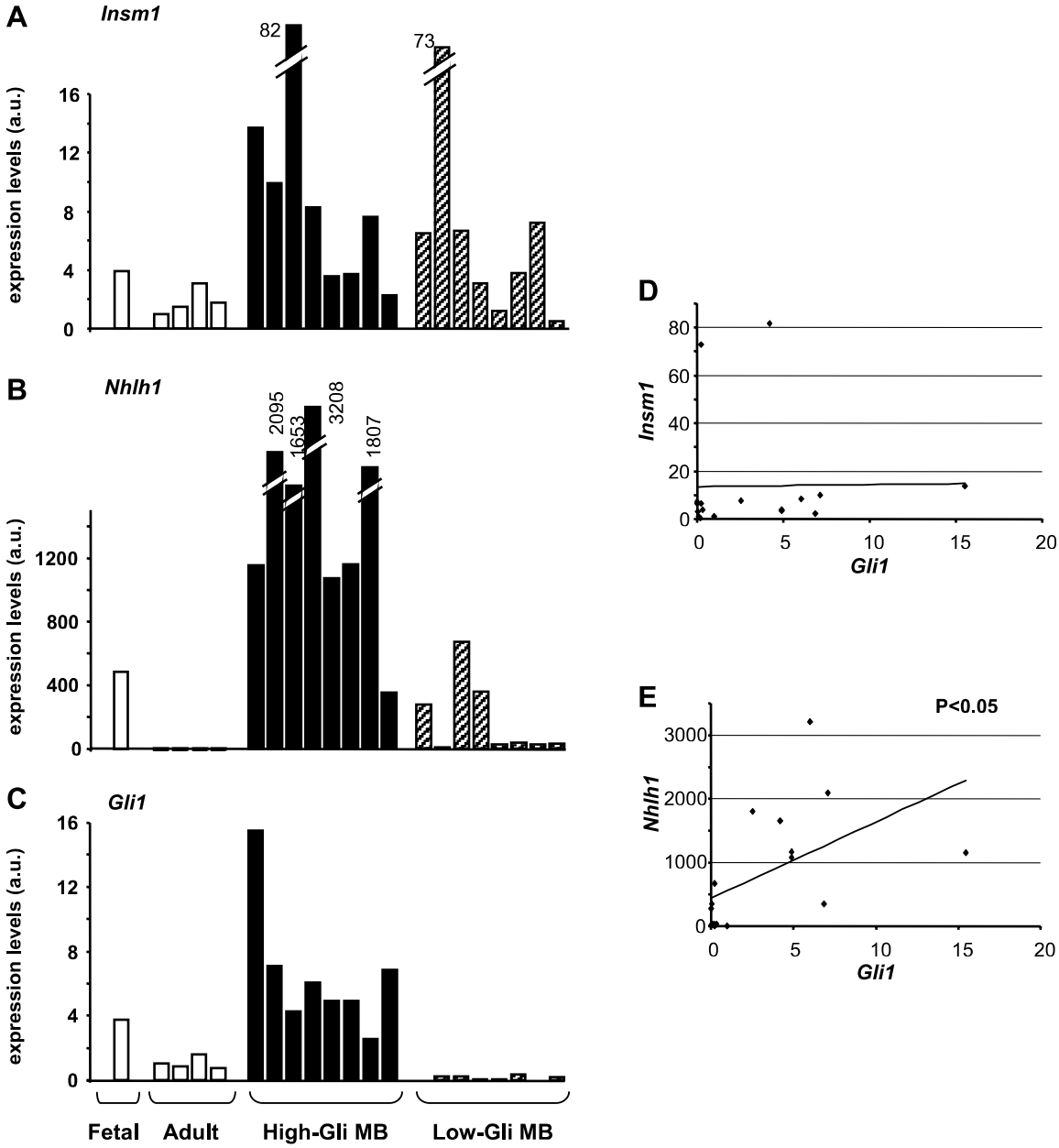

Figure 6. (A–C) *Insm1*, *Nhlh*, and *Gli1* expression levels in ^{high}Gli and ^{low}Gli human MBs compared to fetal and adult control cerebellar tissue. (D–E) Regression curves for *Insm1* (no correlation) and *Nhlh1* ($P < .05$) relative to *Gli1*.

suggesting that it may be involved in GCP proliferation and in the onset of differentiation, but not in the maintenance of the differentiated state [36].

Such a role at the beginning of the differentiation process is also supported by the expression pattern of *Nhlh1* in early differentiating embryonic neuroblasts, where *Nhlh1* is expressed in cells that have just become postmitotic [34]. Interestingly, expression of *Nhlh1* in postmitotic neurons has to be downregulated when the cells have migrated to their final position to allow them to become differentiated [34].

The identification of *Nhlh1* as a novel target of HH pathway, underscores its potential role in the activity of Shh. HH signaling plays a complex role in driving the development of lineage-committed cerebellar GCPs, being mostly responsible for the expansion of an early pool of progenitor cells, which, subsequently, terminally differentiate into postmitotic mature granule cells. HH window of activity is overlapping with the expression of *Nhlh1*, suggesting that this regulatory loop might be involved in the transition from proliferation to early differentiation of progenitor cells.

Whether this process may be relevant for cerebellar tumorigenesis, which stems from disequilibrium between proliferation and differentiation of GCP, is an outstanding question that needs to be appropriately addressed. We have described a deregulated expression of *Nhlh1* in both human and murine MB, with increased levels of both mRNA and protein. Furthermore, we show that *Nhlh1* expression correlates with the strength of HH pathway activity. *Nhlh1* expression may be a feature of HH-dependent tumors in which a deregulation of the signals sustaining the transition from proliferation toward differentiation of GCP would have occurred during the tumorigenic process. These may speculatively include uncoordinated activation *versus* silencing of *Nhlh1* at the proliferation/differentiation transition of early developing GCP or aberrant interactions with other bHLH partners.

In conclusion, by combining gene expression analysis during postnatal development with patterns of gene expression in response to HH signaling pathway and in HH-dependent cerebellar tumors, we have identified novel HH target genes that might provide insights in the molecular events that underlie the neoplastic transformation of neuronal progenitor cells at the critical stage of early development.

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