

The *miR-17~92* cluster collaborates with the Sonic Hedgehog pathway in medulloblastoma

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Medulloblastomas (MBs) are the most common brain tumors in children. Some are thought to originate from cerebellar granule neuron progenitors (GNPs) that fail to undergo normal cell cycle exit and differentiation. Because microRNAs regulate numerous aspects of cellular physiology and development, we reasoned that alterations in miRNA expression might contribute to MB. We tested this hypothesis using 2 spontaneous mouse MB models with specific initiating mutations, *Ink4c*^{-/-}; *Ptch1*^{+/-} and *Ink4c*^{-/-}; *p53*^{-/-}. We found that 26 miRNAs showed increased expression and 24 miRNAs showed decreased expression in proliferating mouse GNPs and MBs relative to mature mouse cerebellum, regardless of genotype. Among the 26 overexpressed miRNAs, 9 were encoded by the *miR-17~92* cluster family, a group of microRNAs implicated as oncogenes in several tumor types. Analysis of human MBs demonstrated that 3 *miR-17~92* cluster miRNAs (*miR-92*, *miR-19a*, and *miR-20*) were also overexpressed in human MBs with a constitutively activated Sonic Hedgehog (SHH) signaling pathway, but not in other forms of the disease. To test whether the *miR-17~92* cluster could promote MB formation, we enforced expression of these miRNAs in GNPs isolated from cerebella of postnatal (P) day P6 *Ink4c*^{-/-}; *Ptch1*^{+/-} mice. These, but not similarly engineered cells from *Ink4c*^{-/-}; *p53*^{-/-} mice, formed MBs in orthotopic transplants with complete penetrance. Interestingly, orthotopic mouse tumors ectopically expressing *miR-17~92* lost expression of the wild-type *Ptch1* allele. Our findings suggest a functional collaboration between the *miR-17~92* cluster and the SHH signaling pathway in the development of MBs in mouse and man.

cerebellum | microRNAs | oncomir1 | granule neuron progenitors

Medulloblastoma (MB), the most common pediatric malignant brain tumor, arises in the cerebellum with at least a subset originating from cerebellar granule neuron progenitor (GNP) cells that fail to properly migrate and differentiate (1, 2). Two inherited cancer syndromes induce MB in humans. Gorlin syndrome is characterized by skeletal abnormalities and large body size in combination with a high incidence of basal cell carcinoma and MB (3). Gorlin patients have mutations in *PATCHED* (*PTCH*), the receptor for Sonic Hedgehog (SHH) (4, 5, 6, 7). SHH is the major mitogen for GNP proliferation (8) and mutations in the SHH pathway induce MB in mice (9, 10). Turcots's syndrome is associated with colon cancer and malignant neuroepithelial brain tumors resulting from mutations of the adenomatous polyposis coli (APC) gene. APC is a member of the wntless (WNT) signaling pathway that regulates the proliferation and fate of neural progenitor cells (11, 12).

Both SHH/PTCH and WNT pathways have also been implicated in sporadic disease. Recent studies of a cohort of 46 St. Jude patients identified 5 distinct MB subgroups, on the basis of mRNA and genomic profiling. Two of these contained mutations that activate the SHH/PTCH (~25%) and WNT (20%) signaling pathways, respectively (13).

The past several years have produced a strong appreciation that changes in noncoding RNAs can also impact tumorigenesis. MicroRNAs are endogenous triggers of the RNAi pathway. These 21–23 nucleotide (nt) RNAs are matured through a 2-step biogenesis mechanism from long, RNA polII transcripts (14). While some microRNAs are encoded from individual transcription units, others reside within introns of protein coding genes or exist as polycistrons that generate multiple microRNAs from a single primary transcript. MiRNAs reduce protein synthesis following recognition of the 3' untranslated region (3'-UTR) of target genes via Watson-Crick base pairing interactions (15). Many miRNAs are conserved throughout evolution, suggesting that they perform fundamental biological functions during development.

Currently 678 human miRNAs and 472 mouse miRNAs have been confidently identified (miRBASE) (16). MicroRNAs have been linked to the initiation, progression, and metastasis of human malignancies, with some species displaying oncogenic and others tumor suppressive potential (17). MiRNAs are often expressed aberrantly in tumors as compared to normal tissues and are likely to contribute to tumorigenesis by dysregulating critical target genes (18).

The *miR-17~92* cluster, also called *Oncomir-1*, was among the first miRNAs to be validated as showing oncogenic potential (19). The cluster has 2 paralogs, *miR-106a~363* and *miR-106b~25*, that are located on different chromosomes and contain individual miRNAs that are highly similar to those encoded by the *miR-17~92* cluster. Despite their similarities, each of these miRNA clusters has distinct functions (20, 21). For example, *miR-17~92* alone is involved in B cell lymphomagenesis and in lung development (21).

We hypothesized that miRNAs might play an important role in normal cerebellar development and that dysregulation of miRNAs might contribute to MB formation. We found that *miR-17~92* is expressed in the developing mouse cerebellum and

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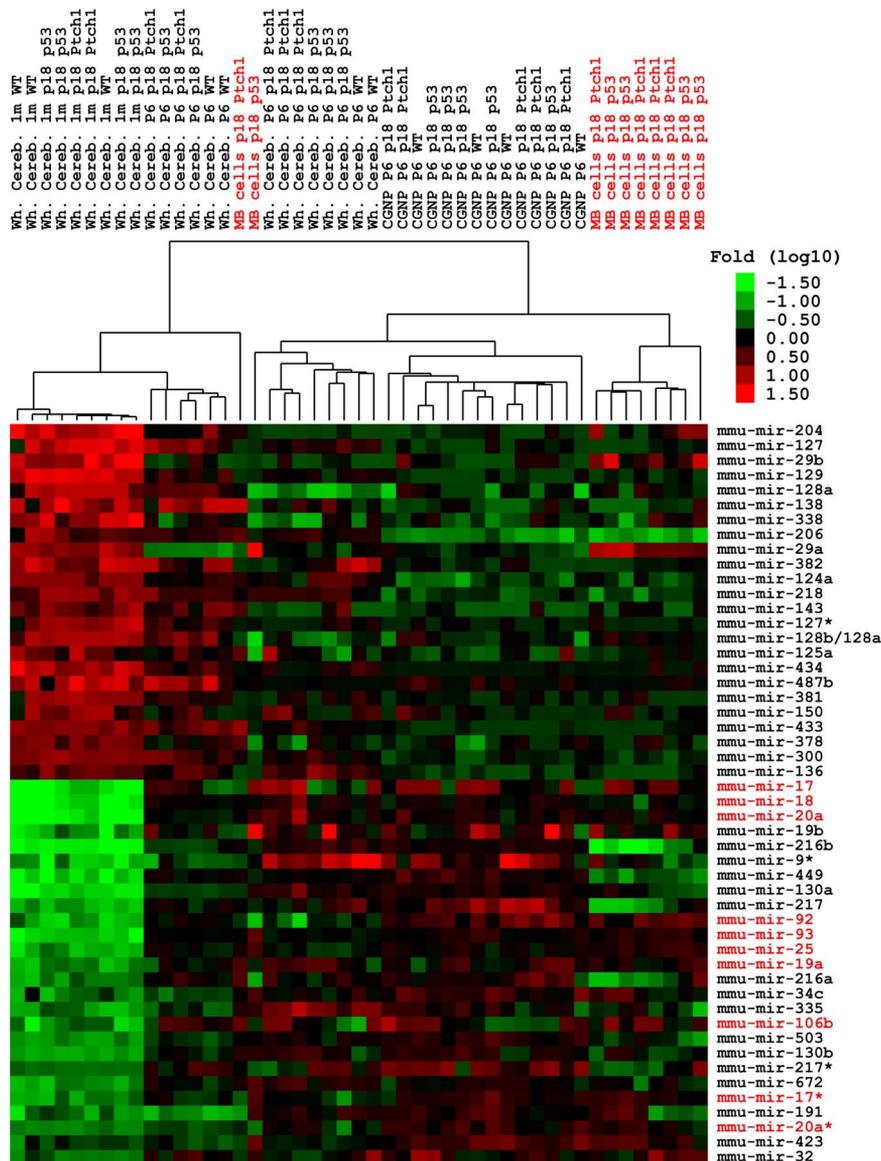


Fig. 1. MiRNA signature during cerebellar development and in mouse MB models. Illumina deep sequencing reveals 50 miRNAs differentially expressed in whole 1-month-old and P6 cerebella, and in purified P6 GNPs from cerebella of wild-type (WT), *Ink4c*^{-/-}; *Ptch1*^{+/-}, *Ink4c*^{-/-}; *p53*^{-/-} mice, or in MBs (red) from both genotypes. Tumor and P6 GNP samples, considered together, were contrasted against adult cerebella and miRNAs were filtered to require a 1% FDR and a 4-fold change between the groups.

in proliferating GNPs but not in postmitotic differentiated neurons. The *miR-17~92* cluster is overexpressed in mouse and human MBs with an aberrantly activated SHH/PTCH signal pathway. Moreover, enforced expression of *miR-17~92* in P6 *Ink4c*^{-/-}; *Ptch1*^{+/-} GNPs induced MBs with complete penetrance after orthotopic transplant into the brains of recipient immunocompromised animals. These results provide the first evidence that the SHH/PTCH signaling pathway and *miR-17~92* functionally interact and contribute to MB development in mice and man.

Results

The *miR-17~92* Cluster Family Is Expressed During Mouse Cerebellar Development and in Mouse MBs. We performed unbiased Illumina deep sequencing and subsequent comparative expression analysis of small RNAs isolated from P6 and 1 month (M) wild-type, *Ink4c*^{-/-}; *p53*^{-/-} or *Ink4c*^{-/-}; *Ptch1*^{+/-} cerebella and GNPs purified from these tissues. These time points were chosen to

coincide with the peak of proliferation of GNPs (P6) and with completion of cerebellar development (1M). miRNAs expression signatures of these tissues were compared with profiles generated from GNP-like tumor cells purified from MBs occurring spontaneously in *Ink4c*^{-/-}; *Ptch1*^{+/-} or *Ink4c*^{-/-}; *p53*^{-/-} mice (22) (Fig. 1).

Twenty-four miRNAs were expressed at higher levels in 1-month-old cerebella relative to P6 GNPs and MBs. These miRNAs might act as tumor suppressors and/or as regulators of genes that block differentiation. Among these was *miR-124a*, which increases during neuronal differentiation under the control of the repressor element-1 silencing transcription factor (REST) complex (23). Also showing greater abundance in postmitotic neurons was *miR-138*, a microRNA that was found to be expressed exclusively in neuronal tissues (24), and *miR-128*, that is highly expressed in the adult brain (25). Several miRNAs from 2 large clusters on mouse chromosome 12 were coordinately upregulated: *miR-300,381,487b,382* and *miR-433,127,434,136*.

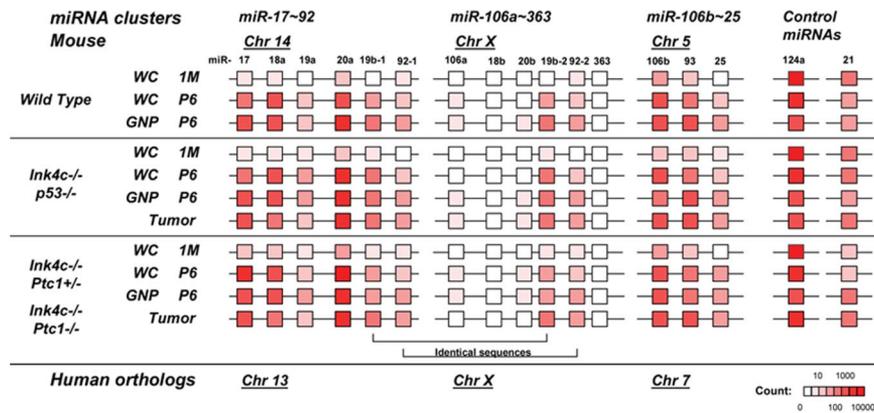


Fig. 2. The *miR-17~92* cluster family is differentially expressed in proliferating GNPs and GNP-like tumor cells compared to postmitotic 1-month-old cerebella. Schematic expression profiles of the 3 miRNA clusters reveals increasing levels of the *miR-17~92* cluster family in proliferating GNPs and GNP-like tumor cells. Expression levels as sequencing read counts are indicated as color-scaled boxes. Each profile as a group was an average of multiple samples. Expression levels of *miR-124a* and *miR-21*, nonmembers of the *miR-17~92* cluster family, are shown as controls. wc, whole cerebellum; WT, wild type.

In contrast, 26 miRNAs showed lower expression in 1-month-old cerebella relative to P6 GNPs and mouse MBs regardless of their genotypes. Among this group might be miRNAs that act as oncomiRs (Fig. 1). Remarkably, 9 members of this group were encoded by the *miR-17~92* cluster and its paralogs (20). We found high expression of the *miR-17~92* and *miR-106b~25* paralog clusters (Figs. 1, 2, and Fig. S1). In contrast, the *miR-106a~363* cluster encoded on mouse and human chromosome X was weakly expressed (Fig. 2 and Fig. S1). Although expression of individual miRNAs within the *miR-106a~363* cluster was 2 times higher in P6 GNPs and MBs compared to 1-month-old cerebella, overall levels remained very low compared to the paralogous clusters (Fig. 2 and Fig. S1). Because the pairs of miRNAs *19b-1* and *19b-2* and miRNAs *92-1* and *92-2* have identical sequences, we could not evaluate whether their expression came from the *miR-17~92* or the *miR-106a~363* cluster. Additionally, the miRNA signature obtained in P6 GNPs and GNP-like tumor cells versus differentiated neurons was similar in wild-type, *Ink4c*^{-/-}; *Ptc1*^{+/-}, and *Ink4c*^{-/-}; *p53*^{-/-} mice. These findings suggest that the miRNA signature that we obtained was characteristic of GNPs and that mutations in *p53*, *Ptc1* and *Ink4c* have little influence on miRNA expression in this cell type under the conditions tested.

The Human MB Subgroup with an Activated SHH/PATCHED Signaling Pathway Overexpresses miRNAs from the *miR-17~92* Cluster. Mouse and human tumors that contain an activated SHH/PTCH pathway share similar global gene expression signatures (26, 13). We therefore tested whether human tumors with an activated SHH/PTCH pathway also upregulate the *miR-17~92* cluster. We performed quantitative real-time reverse transcriptase PCR (QRT-PCR) analysis of miRNAs in 5 normal control cerebella (generously provided by James Olson, Seattle, Washington) and 10 previously characterized human MBs (13) (Fig. 3). RNA isolated from P6 mouse GNPs was used for comparison. Among the MB samples, 5 tumors (MBs 102, 109, 142, 152, and 154) displayed a gene expression signature characteristic of an activated SHH/PTCH pathway; 3 of these (MBs 142, 152, and 154) contained inactivating mutations in *PTCH*. The 5 other human MBs did not display an activated SHH/PTCH signature, nor did they contain mutations in *PTCH*, *SMOOTHENED* (*SMO*), or *SUFU* (13). *miR-19a* ($P = 0.003$), *miR-92* ($P = 0.001$), and *miR-20a* ($P = 0.008$) showed increased abundance specifically in MBs displaying the activated SHH/PTCH pathway signature over those showing a nonactivated SHH pathway ($P = 0.026$) (Fig. 3). These results implicate the *miR-17~92* cluster in the

formation of human MBs that are driven by an aberrant SHH/PTCH pathway and suggest a functional relationship between Patched signaling and *miR-17~92*.

Enforced Expression of *miR-17~92* in GNPs from *Ink4c*^{-/-}; *Ptc1*^{+/-} Mice Accelerates MB in Vivo. To determine whether expression of the *miR-17~92* cluster can lead to oncogenic transformation of primary P6 GNPs, we enforced the expression of the entire cluster in primary GNPs purified from the cerebella of P6 *Ink4c*^{-/-}; *p53*^{-/-} and *Ink4c*^{-/-}; *Ptc1*^{+/-} mice. These mice normally spontaneously develop MBs within 5 months of life. GNPs were infected with mouse stem cell virus (MSCV)-based retroviruses encoding the miRNA cluster along with green fluorescent protein under the control of the SV40 promoter (MLS). Cells were injected immediately into the cortices of immunocompromised naïve recipient mice without prior cell

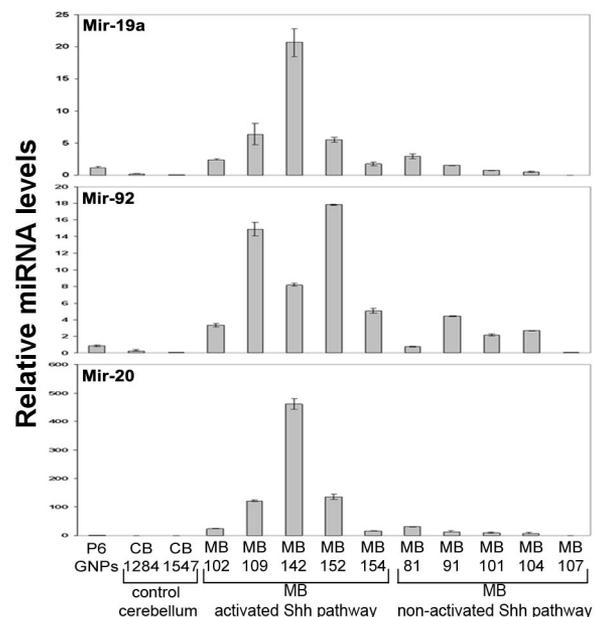


Fig. 3. Human MBs with a SHH/PATCHED gene signature express individual miRNAs from the *miR-17~92* cluster. Quantitative RT-PCR analysis of selected microRNAs from the *miR-17~92* cluster using RNA extracted from human MBs, purified GNPs from P6 mice, and normal human cerebella tissue. MB tumor patient samples were previously molecularly characterized (13).

and might provide not only prognostic value but also clues to the full range of miRNAs that impact human MB.

Functional Interrelationship Between Patched Signaling and the *miR-17~92* Cluster. Purified GNP from P6 *Ink4c*^{-/-}; *Ptch1*^{+/-} mice infected with a GFP-expression control retroviral vector gave rise to rare MBs when orthotopically implanted into recipient animals. These tumors occur with low penetrance. This strongly argues for an impact of stochastic secondary mutations that transform a subset of transplanted cells irrespective of whether they had or had not integrated the viral vector.

In stark contrast, similar GNPs engineered to ectopically express *miR-17~92* by retroviral transfer developed into MBs following transplantation with complete penetrance in 9/9 recipient mice. Interestingly, enforced expression of the *miR-106~25b* paralog, which maps to chromosome X, never gave rise to MBs. This finding is consistent with the fact that this paralog is expressed at low or negligible levels in GNPs and MBs and suggests that lower expression is not determined by the endogenous promoter of this cluster alone but also by posttranscriptional mechanisms (30). *miR-21* has been shown to be overexpressed in many human tumors; however, it was not expressed at all during cerebellum development or in tumors, suggesting specificity in the nature of prooncogenic microRNAs that can contribute to tumor development in the cerebellum. Consistent with the implications of expression profiling, ectopic expression of *miR-21* in GNPs from the cerebella of P6 *Ink4c*^{-/-}; *Ptch1*^{+/-} mice did not induce tumors.

Ink4c; *p53* doubly deficient mice sometimes spontaneously develop MBs. In this genetic context, overexpression of *miR-17~92* in GNPs from the cerebella of P6 mice with this genotype did not induce tumors above this background level upon transplantation of engineered cells into the cortices of recipient mice. Enforced expression of *miR-17~92* did efficiently cooperate with *Patched* loss to induce tumors with complete penetrance, arguing strongly for a functional link between the Patched pathway and this microRNA cluster.

Because we were unable to convincingly show that any of the previously reported targets, including PTEN, were affected at the protein level by ectopic expression of *miR-17~92*, it is not at present possible to propose a specific molecular mechanism by which *miR-17~92* affects the phenotype of GNPs. Our data support the idea that loss of *Patched* is necessary for *miR-17~92* to exert its tumorigenic effect. This could be the result of a direct or indirect interaction between a gene that is activated by SHH signaling, including *N-Myc*, *Gli1*, *Math1*, or other genes, and *miR-17~92*. One could envision several models by which cooperation could occur. For example, (i) *miR-17~92* could repress target(s) that otherwise buffer or counteract the oncogenic signals emanating from the SHH pathway or (ii) *miR-17~92* could amplify the effects of SHH signaling, perhaps by inhibiting a transcriptional repressor of a subset of SHH target genes. In this case, these genes would be activated only when both SHH signaling is on and *miR-17~92* is inhibiting the repressor.

Patched might also impact the transcription of the *miR-17~92* cluster directly, with loss of *Patched* through *Gli1* or other Shh mRNA targets upregulating miRNA expression. While this could explain the specific overexpression of the cluster in tumors with activated SHH signaling, it is difficult to rationalize this hypothesis with the fact that activated SHH signaling is required to uncover the oncogenic effects of *miR-17~92* in GNPs. *miR-17~92* might repress genes that induce cell cycle arrest and/or differentiation or in turn be regulated by such growth arrest and differentiation pathways. In accord with this idea, we previously found that BMP-2, -4, and -7 oppose Shh/Ptch-induced proliferation of GNPs and MBs, by inducing their differentiation and the downregulation of genes in this pathway in mouse and human MBs, but only in tumors with a SHH

signature (31). Thus, BMPs might repress *miR-17~92* levels and thus prevent promotion of MB development.

Whether *miR-17~92* is required for the initiation, progression, and/or maintenance of the disease will require experiments that address the consequences of lost *miR-17~92* function. Unfortunately, primary GNPs cannot be efficiently transfected or electroporated, preventing effective suppression of *miR-17~92* by antagomirs. Experiments using *miR-17~92*-floxed mice in which *miR-17~92* can be conditionally deleted by the Cre recombinase (21) will enable us to test whether *miR-17~92* is essential for GNP proliferation and tumor development when *Ptch1* is mutated.

***miR-17~92* Is a Myc Target.** Myc is one of the most potent oncogenic agent in human cancers. Interestingly, *N-Myc* is expressed at high levels in all MBs from our mouse models, suggesting that *N-Myc* could activate the *miR-17~92* cluster in cerebellar development and MBs. We found that enforced expression of *N-Myc* or *c-Myc* in primary purified GNPs from cerebella of P6 *Ink4c*^{-/-}; *Ptch1*^{+/-} mice induces MBs after orthotopic transplant in the cortices of recipient animals (Table 1). The *miR-17~92* cluster is a direct *c-Myc* target (32) and collaborates with *c-Myc* in B cell lymphoma development (19). In this context, *miR-17~92* may provide cells with a proliferative advantage by preventing cell cycle exit or differentiation and thus collaborate with oncogenic signals to transform GNPs, but would not be oncogenic on its own.

Conclusions

Although the exact mechanism by which Patched and *miR-17~92* collaborate during cerebellar development and in MB formation is still unclear, future studies on *miR-17~92* regulation and the targets that it regulates are warranted, both by expression studies in mouse and human MBs and by functional studies in mice. Our findings indicate that antigomirs to the *miR-17~92* cluster might provide a potential new therapeutic strategy for patients with MBs harboring a constitutively activated SHH/PATCHED signaling pathway.

Methods

MB Patients' Samples. Human MB samples were previously molecularly characterized (13).

Animal Husbandry and Mouse Tumor Samples. Breeding and genotyping of mice were done as reported previously (22). Mice were housed in an accredited facility of the Association for Assessment of Laboratory Animal Care in accordance with National Institutes of Health guidelines. The Animal Care and Use Committee of St. Jude Children's Research Hospital approved all procedures. Mice exhibiting signs of illness (abnormal head movements, cranial expansion, reduced activity, or ataxia) were killed. Tumors were removed and a portion was used for purifying tumor cells. In parallel, a piece of tumor was fixed in 10% neutral-buffered formalin, embedded in paraffin, and subjected to histopathology and immunohistochemistry analysis.

Isolation of Small RNAs and miRNAs, Sequencing, and Data Analysis. Total RNA from GNPs, GNP-like tumor cells, and tissues was extracted using Trizol with an extra chloroform wash and precipitated with isopropanol. Five micrograms (μg) of total RNA were used to amplify and sequence small RNAs as described previously (33), with the introduction of a barcoded multiplexed strategy. RNA adapters ending with a 6-nt barcode followed by a 6-nt constant region were used in the 5'-ligation step. Resulting miRNA libraries were PCR amplified with primers containing Illumina adapter sequences, such that the miRNA would be read in reverse complement followed by the barcode sequence, mixed into pools of 10 libraries with different barcodes, and sequenced on Illumina sequencers. Lack of barcode bias was confirmed by amplifying and sequencing a HeLa sample with the 10 different barcodes. The reads were deconvoluted by barcode, collapsed to unique sequences, aligned to the genome, and annotated. Read counts for miRNA sequences were cubic-spline normalized then log transformed. The R-project software (<http://www.R-project.org>), including Bioconductor were used for data analysis. For identi-

fication of up- and downregulated miRNAs in tumors and P6 GNPs versus adult cerebella, the Bioconductor package linear modeling for microarray (limma) was used to build a model of tumor and P6 GNP samples contrasted against adult cerebella. *P*-values from limma were transformed to *Q*-values for estimation of the false discovery rate (FDR). The set of miRNAs corresponding to a 1% FDR were filtered to require a 4-fold change between the groups. The remaining 50 miRNAs were considered significantly different in the adult cerebella relative to the tumor and P6 GNP samples. For sequences, see *Methods, S1 Text* (Fig. S4). For expression profiling, the normalized read counts for miRNAs encoded by miR-17~92 cluster family were collected, while undetected sequences were scaled to the minimum count of the data set.

GNP and GNP-Like MB Tumor Cell Purification, Retroviral Infection, Orthotopic Injections, and Tumor Cell Cultures. Purification of GNPs from mouse cerebella and MBs were done as described (22). Infection of GNPs and orthotopic injections were carried out as described using 2×10^6 cells (27). To assess the infection efficiency, 3×10^5 infected cells were cultured for 48 h, then fixed and immunostained with anti-GFP. In each experiment, the infection efficiency was at least 30%. MicroRNAs were cloned into a MSCV-SV40 promoter-green fluorescent protein (GFP) vector (19). Tumor cells were purified, cultured, and treated with cyclopamine, as previously described (31).

Quantitative Real-Time PCR. Quantitative real-time reverse transcriptase PCR (QRT-PCR) for *Ptch1*, *Math1*, and *Gli1* on RNA extracted from purified GNP-like tumor cells was done as described (22). QRT-PCRs for *miR-19a*, *miR-92*, and *miR-20* were performed using Applied Biosystems TaqMan MicroRNA assays according to manufacturer's recommendations (ABI, Fos-

ter City, CA). Briefly, cDNAs were synthesized from total RNA using miRNA-specific primers according to the TaqMan MicroRNA reverse transcription protocol. Reverse transcription was performed using the following program: 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C, and then held at 4 °C. Q-PCR on the cDNAs was performed using a 7900HT sequence detection system and the TaqMan Universal MasterMix reagents. Cycling conditions were 10 min at 95 °C, followed by 40-cycle amplification for 15 sec at 95 °C and 1 min at 60 °C. The internal *RNU6B* RNA levels were used for normalization. Each measurement was performed in triplicate. Data were analyzed with SDS version 2.0 software (ABI).

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