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## Functional Genomics Identifies Drivers of Medulloblastoma Dissemination

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### Abstract

Medulloblastomas are malignant brain tumors that arise in the cerebellum in children and disseminate via the cerebrospinal fluid to the leptomeningeal spaces of the brain and spinal cord. Challenged by the poor prognosis for patients with metastatic dissemination, pediatric oncologists have developed aggressive treatment protocols, combining surgery, craniospinal radiation, and high-dose chemotherapy that often cause disabling neurotoxic effects in long-term survivors. Insights into the genetic control of medulloblastoma dissemination have come from transposon insertion mutagenesis studies. Mobilizing the Sleeping Beauty transposon in cerebellar neural progenitor cells caused widespread dissemination of typically nonmetastatic medulloblastomas in *Patched*<sup>+/-</sup> mice, in which Sonic Hedgehog (Shh) signaling is hyperactive. Candidate metastasis genes were identified by sequencing the insertion sites and then mapping these sequences back to the mouse genome. To determine whether genes located at transposon insertion sites directly caused medulloblastomas to disseminate, we overexpressed candidate genes in Nestin<sup>+</sup> neural progenitors in the cerebella of mice by retroviral transfer in combination with Shh. We show here that ectopic expression of *Eras*, *Lhx1*, *Ccrk*, and *Akt* shifted the *in vivo* growth characteristics of Shh-induced medulloblastomas from a localized pattern to a disseminated pattern in which tumor cells seeded the leptomeningeal spaces of the brain and spinal cord.

### Keywords

medulloblastoma; metastasis; leptomeningeal dissemination

### Introduction

Medulloblastomas are malignant brain tumors that originate from neural progenitor cells in the developing cerebellum. A powerful predictor of short survival times for pediatric patients is the presence of metastasis (1). A defining characteristic of metastasis in

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medulloblastoma is the proclivity of tumor cells to disseminate via the cerebrospinal fluid (CSF) to the leptomeninges of the brain and spinal cord. This pattern distinguishes medulloblastomas from tumors originating in other organs, which metastasize through the bloodstream or lymphatic channels. The pressing need to reduce the risk of metastasis has driven the development of aggressive treatment protocols, combining maximum surgical resection, craniospinal radiation, and multidrug chemotherapy [reviewed in (2)].

Because the prospect for long-term survival is so poor once leptomeningeal dissemination has occurred, radiation to the entire neuraxis is an indispensable part of medulloblastoma treatment regimens for children older than three years. After treatment, however, children are at high risk for developing cognitive impairment, skeletal growth retardation, endocrine dysfunction, and behavioral disturbances later in life (3, 4). Therefore, therapies that target metastasis specifically will likely protect patients from some of these treatment-related side effects.

An emerging concept in pediatric oncology is that medulloblastomas comprise a diverse set of tumors, in which different subgroups arise by transformation of neural progenitor cells, responding to different molecular signaling pathways. Large-scale gene expression profiling studies of patient tumor specimens have shown that the Sonic Hedgehog (Shh) signaling pathway is activated in 25–30% of medulloblastomas (5–7).

Animal models of medulloblastoma, created using genetically engineered mice, have shown conclusively that activating the Shh signaling pathway in the cerebellum during early postnatal development can induce medulloblastoma formation. Several different methods of activating the Shh pathway have been reported, including (a) targeted deletion of the *Patched* gene, which encodes the inhibitory receptor for Shh (8), (b) ectopic expression of *Shh* by retroviral transfer (9, 10), and (c) transgenic overexpression of *Smoothed*, a positive effector of Shh signaling (11). With the exception of a homozygous version of the ND2:SmA1 model, in which expression of a constitutively activated allele of the *Smoothed* gene is driven by the *neuroD2* gene promoter (12), leptomeningeal dissemination has not been reported in mouse models of Shh-driven medulloblastoma.

The tight correlation between metastasis and poor prognosis for medulloblastoma patients heightens the need to understand the genetic determinants of leptomeningeal dissemination. Insights into the genetic control of medulloblastoma dissemination have come from experiments in which the Sleeping Beauty (SB) transposon system was used to induce insertional mutations in granule neuron precursors (GNPs) in the developing cerebellum in mice. GNPs are lineage-restricted progenitor cells that can transform into medulloblastomas in response to hyperactive Shh signaling (13–15).

The SB transposon system (Fig. 1) is a powerful tool for cancer gene discovery in mice [reviewed in (16)]. In the presence of a transposase enzyme, the SB transposon is excised from genomic DNA and inserted throughout the genome at sites containing TA dinucleotides. A transposon integration site present in the genome at a significantly high frequency is called a common insertion site (CIS). Tumors can arise if the transposon activates proto-oncogenes or inactivates tumor suppressor genes located at the integration sites (transposon insertion mutagenesis).

To discover genes that promote spinal leptomeningeal dissemination in Shh-driven medulloblastomas, Wu *et al* (17) expressed the SB transposase selectively in *Math1*<sup>+</sup> GNPs in *Patched*<sup>+/-</sup> mice, which develop localized, nonmetastatic medulloblastomas at an incidence of 39% by 8 months of age. In sharp contrast, 97% of *Patched*<sup>+/-</sup> mice in which the SB transposon was mobilized by driving expression of the SB transposase from the *Math1* gene promoter developed medulloblastomas that metastasized widely throughout the

spinal leptomeninges by 10 weeks of age. Candidate metastasis genes were identified by sequencing the insertion sites in tumor-bearing animals and then mapping these sequences back to the mouse genome. In this way, the investigators identified *gene-centric common insertion sites* (gCISs), in which transposon insertions occurred significantly more frequently than the background rate. In 139 matched pairs of primary/metastatic tumors from *Patched*<sup>+/-</sup> mice, 359 gCISs were identified in the primary brain tumors, 285 in the spinal metastases, and 60 in both primary tumor and spinal metastasis from the same animals. To determine whether gCIS-associated genes can promote dissemination of tumor cells from Shh-induced medulloblastomas to the leptomeningeal spaces of the brain and spinal cord, we expressed four candidate genes (*Eras*, *Lhx1*, *Ccrk*, and *Akt*) in neural progenitor cells in the cerebellum of mice by retroviral transfer in combination with *Shh*.

## Materials and Methods

### Transgenic mice

The use of mice in this study was approved by the Institutional Animal Care and Use Committee of the University of Utah. Production of the *Ntv-a* mouse line, in which expression of the *tv-a* transgene is driven by promoter/enhancer sequences of the *Nestin* gene, has been described previously (18). Because of the breeding strategy used to introduce the transgene, *Ntv-a* mice are hybrids composed of the following genetic strains: C57BL/6, BALB/C, FVB/N, and CD1.

### Retroviral vector construction

Construction of RCAS-Shh, which contains an in-frame, carboxy-terminal epitope tag consisting of six repeats of the influenza virus hemagglutinin (HA) epitope, was described previously (10). RCAS-Akt transfers an oncogenic allele of *Akt* (Akt-Myr- $\Delta$ 11-60) that contains an amino-terminal myristylation signal, which enhances the affinity of the Akt protein for the plasma membrane (19, 20). The cDNA clones for *Eras* (mouse), *Lhx1* (mouse), and *Ccrk* (human) were obtained from the American Type Culture Collection (Manassas, VA), where they were deposited by the Integrated Molecular Analysis of Genomes and their Expression (IMAGE) consortium (<http://image.hudsonalpha.org>). RCAS vectors were prepared by ligating a PCR-generated cDNA corresponding to the complete coding sequence into the parent retroviral vector RCASBP(A) (21). RCAS vectors for *Eras*, *Lhx1*, and *Ccrk* each contained an internal ribosome entry site (IRES), coupled to the *Aequorea coerulea* green fluorescent protein (GFP), for tracking the cellular localization of the expressed proteins. To produce live virus, we transfected plasmid versions of RCAS vectors into immortalized chicken fibroblasts (DF-1 cells) and allowed them to replicate in culture.

### In vivo somatic cell gene transfer in transgenic mice

To induce medulloblastomas in mice, we used a version of the RCAS/*tv-a* somatic cell gene transfer system to transfer and express the *Shh* gene in Nestin<sup>+</sup> neural progenitor cells in the cerebellum. This system uses a replication-competent, avian leukosis virus, splice acceptor (RCAS) vector, derived from the subgroup A avian leukosis virus (ALV-A), and a transgenic mouse line (*Ntv-a*) that produces TVA (the cell surface receptor for ALV-A) under control of the *Nestin* gene promoter (18). Nestin is an intermediate filament protein that is expressed by multipotent neural progenitor cells. When mammalian cells are transduced with RCAS retrovirus vectors, the newly generated provirus integrates into the host cell genome, and the transferred gene is expressed as a spliced message under control of the constitutive retroviral promoter (long terminal repeat sequence). RCAS-transduced mammalian cells do not produce infectious virus because mRNA splicing eliminates the retroviral genes that are necessary for viral replication.

To transfer genes via RCAS vectors, we injected retrovirus packaging cells (DF-1 cells transfected with and producing recombinant RCAS retrovirus) into the lateral cerebellum from an entry point just posterior to the lambdoid suture of the skull (bilateral injections of  $10^5$  cells in 1–2  $\mu$ l of phosphate buffered saline). For experiments, in which simultaneous transfer of two genes was the goal, cell pellets were prepared by mixing equal numbers of both retrovirus-producing cells. We injected mice within 72 hours after birth because the number of Nestin-expressing neural progenitor cells decreases progressively afterward. The mice were sacrificed as soon as they showed signs of increased intracranial pressure, indicated by enlarging head circumference (a sign of hydrocephalus), gait ataxia, or failure to thrive. Asymptomatic mice were sacrificed 4 months after injection. The brains were fixed in formalin, and quartered by parallel incisions in the coronal plane. To identify dissemination of tumor cells to the spinal leptomeningeal space, we fixed whole spinal column preparations in formalin for 48–72 hr and then removed the spinal cord by microdissection. Brain and spinal cord specimens were embedded in paraffin and sectioned for histochemical analysis.

### Immunocytochemistry and microscopy

Tissue sections were cut 4  $\mu$ m thick, mounted on glass slides, deparaffinized with toluene, hydrated through a descending series of ethanol, autoclaved in a citrate-based antigen retrieval solution (Vector Laboratories, Burlingame, CA) for 5 min, and cooled to room temperature. Sections were then treated with  $H_2O_2$  (1% v/v) for 10 min to quench endogenous peroxidase activity and washed with phosphate-buffered saline. After immersion in normal horse serum (2%), sections were incubated with primary antibody in a humid chamber at 4°C overnight. Immunoreactive staining was visualized using a biotin-free reporter enzyme staining system (ImmPRESS, Vector Laboratories, Burlingame, CA), which utilizes a micropolymer of peroxidase and affinity-purified secondary antibodies. Diaminobenzidine was used as the chromogenic substrate and toluidine blue as a nuclear counterstain. We used the following antibodies from the indicated commercial sources: mAbF7 (1:50)—HA (Santa Cruz Biotechnology; Santa Cruz, CA); mAb3580 (1:500)—GFP (Chemicon; Temecula, CA). Tissue sections were visualized using a Zeiss Axiovert 200 microscope and photomicrographs were captured using an AxioCam high-resolution CCD camera and Axiovision imaging software (Carl Zeiss International, Germany).

### Expression profiling and molecular subgrouping of human medulloblastomas

Human primary medulloblastomas (n=103) were profiled on Affymetrix Genechip Human Exon 1.0ST arrays at The Centre for Applied Genomics (TCAG, [www.tcag.ca](http://www.tcag.ca); Toronto, Canada). Expression analysis was performed, using Affymetrix Expression Console (Version 1.1), as previously described (22). Additional, publically available medulloblastoma expression data sets (n=187) were obtained from NCBI Gene Expression Omnibus and used to validate our findings (6, 23). Subgrouping of tumors was performed using an 84-gene expression classifier (7).

## Results

### Shh-induced medulloblastomas are localized (nonmetastatic) tumors

Our objective was to use the RCAS/*tv-a* somatic cell gene transfer system to determine whether ectopic expression of genes associated with SB gCISs in *Patched*<sup>+/-</sup> mice could promote spinal leptomeningeal dissemination in mice bearing Shh-induced medulloblastomas. First, we injected a control group of newborn *N/tv-a* mice with RCAS-Shh and examined hematoxylin and eosin (H&E)-stained sections of brain and spinal cord during a 4-month observation period. We found tumors in the cerebellum in 27 of 64 mice (42%), an incidence that was consistent with our previous studies, which showed that 15–

39% of mice developed brain tumors during 3 months of observation (19, 24–26). Microscopically, the tumors resembled the classic histological subtype of human medulloblastomas, which is characterized by homogeneous sheets of densely packed cells containing carrot-shaped, hyperchromatic nuclei and scant cytoplasm (Fig. 2A). Careful examination of spinal cord sections showed small clusters of tumor cells attached to the leptomeninges of the cord or transiting spinal nerves in only 3 of 64 mice examined (5%) (Fig. 2B). Thus, dissemination of tumor cells to the spinal leptomeningeal space occurred in only 11% of the 27 mice in which tumors had formed in the brain (Table 1). We concluded that the background level of spinal leptomeningeal dissemination in *Shh*-induced medulloblastomas was sufficiently low to use our mouse model as a platform for identifying more potent metastasis-inducing genes.

### **Eras, Lhx1, and Ccrk are metastasis-inducing oncogenes**

To identify the strongest driver genes of medulloblastoma leptomeningeal dissemination, we first focused on the 285 gCISs that were present either exclusively in the spinal metastatic tumors or in both the primary tumors and matched metastases. The objective of this criterion was to focus on genes whose expression conferred a growth advantage to tumor cells in the microenvironment of the spinal cord. Conceivably, genetic selection pressure would then lead to the clonal expansion of gCIS-containing cells that were adapted to this new milieu.

Second, we selected gCISs in which (a) the SB transposon had integrated 5' of exon I or into intron I, and (b) the murine stem cell virus (MSV) promoter of the SB transposon pointed to the direction of gCIS-associated gene transcription. We used these criteria to identify genes whose transcription might be activated by the integrated transposon. We selected such genes, which were likely to be metastasis-promoting oncogenes, because the RCAS/*tv-a* system was designed to transfer and express dominantly activating genes. We did not test medulloblastomas from SB mice for altered expression of the gCIS. Although the correlation between transposon insertion and gCIS expression has not been analyzed comprehensively, studies of SB-induced T-cell lymphomas and squamous cell carcinomas indicate that a close correlation exists (27, 28). We also selected gCISs that occurred in the spine tumors from 3 different mice to minimize the effect of random integration. Of the 285 metastasis gCISs, 32 met this second set of criteria.

Third, we selected gCISs (n=20) that had a coding sequence <3000 base pairs to assure efficient transfer and expression of genes via RCAS retroviral vectors in mice. We focused initially on three genes for which there was published literature that strongly supported a role for the encoded protein in cancer biology: *Eras* (embryonic stem cell-expressed Ras), *Lhx1* (LIM-class homeobox gene 1), and *Ccrk* (cell cycle-related kinase).

To determine whether *Eras*, *Lhx1*, and *Ccrk* could directly cause *Shh*-induced medulloblastomas to metastasize, we used the RCAS/*tv-a* system to transfer and express each gene in Nestin<sup>+</sup> neural progenitor cells in the cerebella of newborn mice, in combination with *Shh*. Our results demonstrated that *Eras*, *Lhx1*, and *Ccrk* increased the incidence of spinal leptomeningeal dissemination, as a percentage of mice with histologically verified tumors in the cerebellum, from a baseline of 11% (*Shh* alone) to 29% (*Shh+Eras*), 33% (*Shh+Lhx1*), and 43% (*Shh+Ccrk*) (Table 1). In addition to increasing the incidence of spinal leptomeningeal dissemination, *Eras*, *Lhx1*, and *Ccrk* increased the thickness of tumor cell nodules that were attached to the spinal cord and nerves five- to eightfold (Figs. 2C, E). The mean cross-sectional area of spinal leptomeningeal nodules in mice bearing *Shh*-induced medulloblastomas (0.026 mm<sup>2</sup>) was increased by the addition of *Eras* (0.157 mm<sup>2</sup>), *Lhx1* (0.207 mm<sup>2</sup>), and *Ccrk* (0.134 mm<sup>2</sup>) ( $P=0.02$  by ANOVA).



Importantly, expression of *Eras*, *Lhx1*, or *Ccrk* did not significantly increase the incidence of tumor formation in the brain compared with that of *Shh* alone, indicating that these genes were specific drivers of metastasis, not merely initiators of tumor formation (Table 1). To verify that the tumor cells expressed the genes that we transferred by RCAS vectors, we demonstrated specific immunostaining with an antibody directed against GFP, which was transcribed in tandem with the inserted oncogene through an IRES sequence (Figs. 2D, F).

In the clinical setting, metastasis of medulloblastomas to the spinal column is accompanied by spread of tumor cells to the leptomeninges of the cerebellum and forebrain. Accordingly, we observed in mice that medulloblastomas induced by *Shh* in combination with *Eras*, *Lhx1*, or *Ccrk* showed extensive dissemination of tumor cells to brain leptomeningeal spaces that were not contiguous with the primary tumor site in the cerebellum. Figure 3 shows examples of tumor dissemination to the brain stem (Figs. 3A, B), hippocampal fissure (Figs. C, D), and the subependymal space of the lateral ventricles (Fig. 3E). We scored brain sections from all tumor-bearing mice for invasiveness, which we defined as (a) tumor on brain sections remote from the cerebellum or (b) local extension into the adjacent fourth ventricle (Fig. 3F). The percentage of *Shh*-induced medulloblastomas showing invasiveness (48%) was increased by the addition of *Eras* (88%), *Lhx1* (83%), and *Ccrk* (74%) ( $P=0.003$  by  $\chi^2$  contingency test).

Despite the marked effect on promoting spinal leptomeningeal dissemination, ectopic expression of *Lhx1* and *Ccrk* did not reduce overall survival of mice compared with the *Shh* control group (Fig. 4A). *Eras* showed a trend toward shorter survival time ( $P=0.086$  by log-rank test). We attribute this to the fact that almost all of the tumor-bearing mice were sacrificed because of symptoms due to brain compression by the primary tumor or to obstructive hydrocephalus, which we did not attempt to treat. Therefore, the mice did not live long enough to succumb to metastatic disease. This experimental scheme contrasts sharply with current clinical practice, in which aggressive surgical resection of the primary tumor and decompression of hydrocephalus are essential prerequisites for progression-free survival in patients.

### Expression of ERAS, LHX1, and CCRK is increased in aggressive subgroups of human medulloblastomas

Given the identification of *Eras*, *Lhx1*, and *Ccrk* in a large, unbiased genetic screen for metastasis genes using the SB transposon system and considering our supportive observation of *Shh*-induced medulloblastomas transitioning from a localized to a disseminated growth pattern, we next focused on the expression of our candidate genes in a large cohort of primary human tumors. We compared relative expression levels of *ERAS*, *LHX1*, and *CCRK* in 103 human medulloblastoma specimens, which had each been assigned to one of the four distinct, nonoverlapping medulloblastoma subgroups (WNT, SHH, Group 3, and Group 4) based on their gene expression profiles (7, 29). Our analysis demonstrated that expression of *ERAS* and *CCRK* was higher in Group 3 tumors (Fig. 5A–B) and expression of *LHX1* was higher in Group 4 (Fig. 5C), compared with the undivided set of 103 tumors. The association between *CCRK* and *LHX1* expression and tumor subgroup was recapitulated in a second, independently generated data set from 187 medulloblastomas in which *ERAS* had not been assessed (Supplementary Fig. 1).

Previously published work showed that metastasis (defined by the presence of microscopic tumor cells in the CSF, radiographically detected leptomeningeal dissemination, or metastasis outside of the central nervous system) was significantly more common in Group 3 (46.5%) and Group 4 (29.7%) than in the WNT (17.9%) and SHH (19.1%) subgroups (7). In keeping with the above differences in metastasis prevalence, analysis of large cohorts of patients assigned to these subgroups has shown that mean survival times decline

progressively in the following order: WNT>SHH>Group 4>Group 3. The fact that mRNA levels of *ERAS*, *LHX1*, and *CCRK* were elevated in tumor subgroups that show a high rate of metastasis and short patient survival times indicates that these genes promote aggressive growth in human medulloblastomas, as they do in experimentally induced tumors in mice. Group 3 and Group 4 medulloblastomas might originate from a completely different precursor cell population than SHH tumors.

### PI3K signaling promotes spinal leptomeningeal dissemination in Shh-induced medulloblastomas

The SB transposon mutagenesis study showed that *Pten*, *Akt2*, *Igf2*, and *Pik3r* were metastasis gCIS-associated genes, thus implicating the PI3K signaling pathway in medulloblastoma metastasis (17). Among these genes, *Akt2* and *Pik3r* met the previously described selection criteria for metastasis-inducing oncogenes. The SB insertions most likely disrupted transcription of the *Pten* tumor suppressor gene. Several lines of evidence support the idea that PI3K signaling cooperates with Shh signaling to stimulate medulloblastoma growth. Derepression of the PI3K pathway by loss of *Pten* increased tumor formation in mice carrying an oncogenic allele of *Smoothed* (30). We reported previously that activation of PI3K signaling by insulin-like growth factor-II increased the incidence of Shh-induced medulloblastoma formation and promoted dissemination of tumor cells to the leptomeninges of the brain (17, 19). To determine whether activating the PI3K pathway could directly cause medulloblastoma cells to seed the spinal leptomeninges, we used an RCAS retroviral vector to transfer an activated, transforming allele of *Akt* (Akt-Myr- $\Delta$ 11-60) to Nestin<sup>+</sup> cerebellar progenitors in *Ntv-a* mice and examined spinal cord sections using the methods described above. Mice injected with RCAS-Shh+RCAS-Akt developed tumors in the cerebellum in 56% of cases ( $P=0.16$  compared with RCAS-Shh). The enhancing effect of *Akt* on primary brain tumor induction was less than that which we reported previously (19). This difference is likely due to the longer observation time in the current study (4 vs. 3 months), during which accumulation of secondary mutations can accelerate the growth of Shh-induced tumors. Nevertheless, the incidence of spinal leptomeningeal dissemination was fourfold higher in medulloblastomas induced by *Shh*+*Akt* compared with *Shh* alone ( $P=0.016$ ) (Table 1). The fact that overall survival was significantly reduced in mice bearing tumors induced by *Shh*+*Akt* compared with *Shh* alone ( $P=0.0024$ ; Fig. 4B) indicated that PI3K pathway activation promoted not only the dissemination of tumor cells to the spinal leptomeningeal space, but also more aggressive tumor growth in the brain. No association was found, however, between mRNA levels of any one of the three human *AKT* genes and aggressive tumor subgroup.

### Discussion

Using a genetically engineered mouse model of Shh-induced medulloblastoma, we show here that ectopic expression of *Eras*, *Lhx1*, and *Ccrk* shifted the *in vivo* growth characteristics from a localized to a disseminated pattern, in which tumor cells seeded the leptomeninges of the brain and spinal cord. Expression of these genes did not increase the incidence of tumor formation in the brain, rather resulted in the acquisition of metastatic traits that enabled medulloblastoma cells to detach from the tumor mass, intravasate into the CSF, and reimplant in the leptomeningeal space. Additionally, we explored the role of PI3K signaling in medulloblastoma pathogenesis and report a previously unknown link between PI3K pathway activation by Akt and spinal leptomeningeal dissemination.

While the genes investigated in this study have previously been implicated in cancer biology, the specific mechanisms through which they contribute to metastasis remain uncertain. A growing body of research provides clues to their function. *Eras*, a membrane-localized, GTP-binding protein, is believed to mimic the constitutively active Ras

oncoprotein, based on structural similarities. Furthermore, Eras binds the catalytic subunit of PI3K and activates the PI3K signal transduction pathway, mediating tumor cell invasiveness (31). Importantly, Eras is frequently expressed in human gastric carcinomas, and tumor immunoreactivity is associated with an increased risk of metastasis (32).

Lhx1 is a transcription factor that is crucial for normal kidney morphogenesis and a positive regulator of tumor cell motility, migration, and invasiveness (33). Evidence from renal cell carcinoma cells suggests a positive feedback mechanism through which Shh signaling increases Lhx1 levels, supporting its oncogenic role in medulloblastoma (34).

Ccrk is a serine-threonine kinase that promotes cell cycle progression in mammalian cells (35). Overexpression of Ccrk transforms human immortalized liver cells, and this process is dependent upon  $\beta$ -catenin signaling (36). Ccrk has oncogenic properties beyond promoting cell cycle progression, insofar as depletion of Ccrk by RNA interference fails to cause the expected cell cycle arrest (37).

The epithelial-mesenchymal transition (EMT) is a process by which carcinoma cells lose their epithelial growth characteristics to become detached and invasive, thus acquiring metastatic properties [reviewed in (38)]. A large body of literature has implicated PI3K signaling as paramount to EMT, with oncogenic growth factors, like hepatocyte growth factor and insulin-like growth factors, triggering EMT through PI3K pathway activation [reviewed in (39)].

While the contributions of an EMT-like process to medulloblastoma leptomeningeal dissemination remain speculative, the antiapoptotic effect of PI3K signaling could confer to tumor cells the survival traits necessary to ultimately colonize the spinal leptomeninges. Specifically, PI3K signaling is a key component of the protective response of cells against anoikis, a version of apoptosis triggered when cells become detached from a solid surface (40). PI3K pathway activation is emerging as an essential adaptive response in metastasizing cancer cells as they are shed from the central tumor mass [reviewed in (41)]. PI3K signaling might also promote aggressive growth of medulloblastomas by directly stimulating the Shh signaling pathway through a cross-talk mechanism similar to that reported recently in esophageal adenocarcinoma (42).

Although medulloblastomas can metastasize outside of the nervous system, their usual mode is to disseminate along CSF channels to spinal and intracranial leptomeninges. Our knowledge of leptomeningeal metastasis is rudimentary. The fact that SB transposon insertions were found in *Lhx1* and *Ccrk* in spinal metastatic tumors, but not in the primary brain tumors, suggests that misexpression of these genes conferred a selective growth advantage to tumor cells in the spinal microenvironment. For that reason, *Lhx1* and *Ccrk* are analogous to metastasis virulence genes in the invasion–metastasis cascade of epithelial cancers [reviewed in (43)]. *Eras* and the PI3K pathway genes *Pten*, *Akt2*, *Igf2*, and *Pik3r* had transposon insertions in both spine tumors and brain tumors, indicating, by analogy to metastasis initiation and progression genes in the invasion–metastasis model, possible roles in tumor cell invasiveness, detachment, and resistance to anoikis.

By quantifying SB transposon insertions in medulloblastomas and corresponding spinal metastases, Wu *et al* (17) showed that metastatic tumors can originate from cells that make up only a minor subclone in the primary tumor in the cerebellum. This concept has important therapeutic implications, insofar as treatments aimed at molecular targets in the primary tumor might not be effective against genetically divergent metastatic tumors. We detected no apparent difference in the percentage of transgene-expressing cells in the cerebellar tumors compared with the spinal metastases in our mouse model system, probably because we expressed the metastasis-driving transgenes concurrently with the tumor-



initiating *Shh* gene in the cerebellum. The fact that the levels of *ERAS*, *LHX1*, and *CCRK* mRNA are increased in aggressive subgroups of human medulloblastomas supports the idea that some of the genetic events that drive leptomeningeal dissemination are present in the original tumors.

We used a highly stringent set of criteria for selecting testable metastasis genes. This approach was taken to design a tractable experimental plan. The fact that hundreds of gCISs were found in SB transposon-induced metastases shows that the genetic landscape of medulloblastoma dissemination is very complex. Nevertheless, our results indicate that Shh-induced medulloblastomas can start down a path of disseminated growth by addition of only a single gene. It is not known in individual patients how many different genes initiate and maintain metastasis. The answer to this question will require genomic analysis of medulloblastoma cells that have metastasized to the spinal leptomeningeal space, a project that is hindered by the fact that surgical excision of metastatic tumors is rarely indicated in patient treatment plans.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## ABBREVIATIONS

|             |  |
|-------------|--|
| <b>Shh</b>  | Sonic Hedgehog   |
| <b>ALV</b>  | avian leukosis virus   |
| <b>RCAS</b> | replication-competent, avian leukosis virus, splice acceptor |
| <b>PI3K</b> | phosphatidylinositol 3-kinase                                |
| <b>gCIS</b> | gene-centric common insertion site                           |
| <b>GNP</b>  | granule neuron precursor                                     |
| <b>GFP</b>  | green fluorescent protein                                    |
| <b>Eras</b> | embryonic stem cell-expressed Ras                            |
| <b>Lhx1</b> | LIM class homeobox-1   |
| <b>Ccrk</b> | cell cycle-related kinase                                    |
| <b>EMT</b>  | epithelial-mesenchymal transition                            |

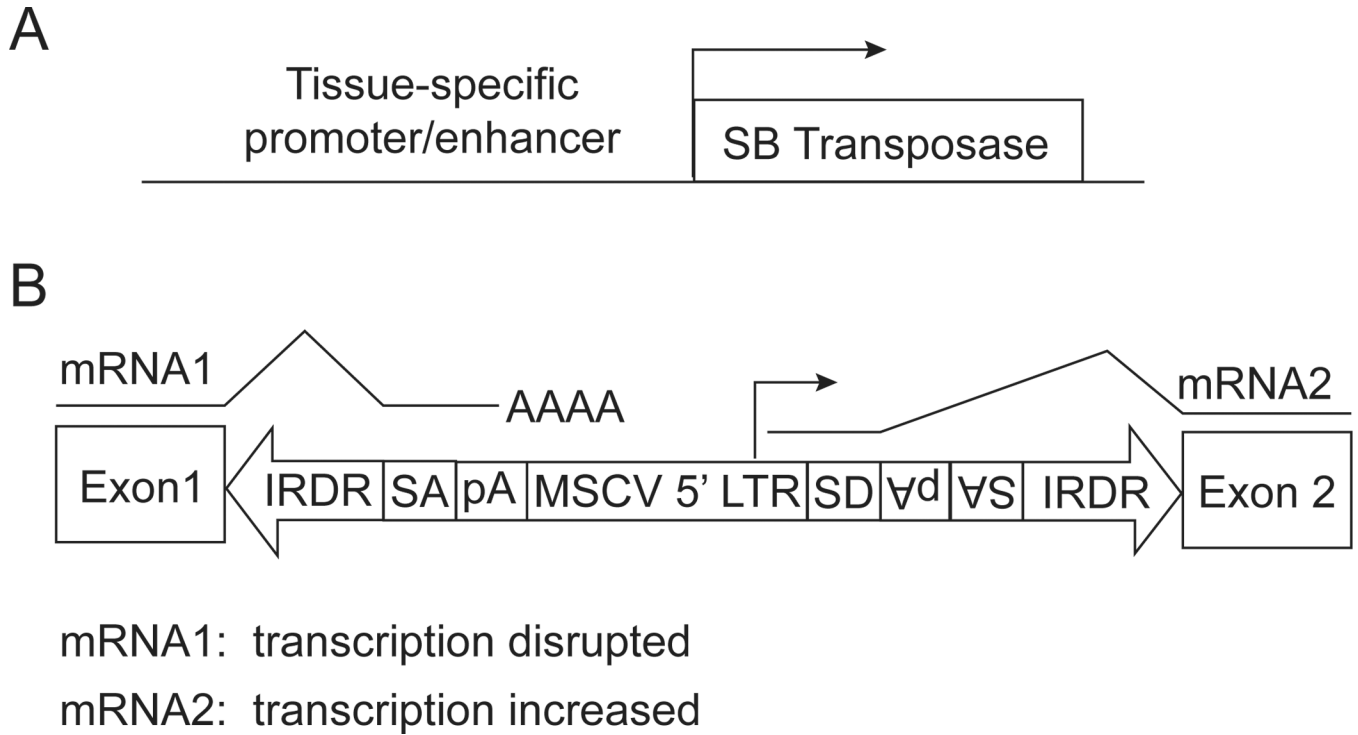
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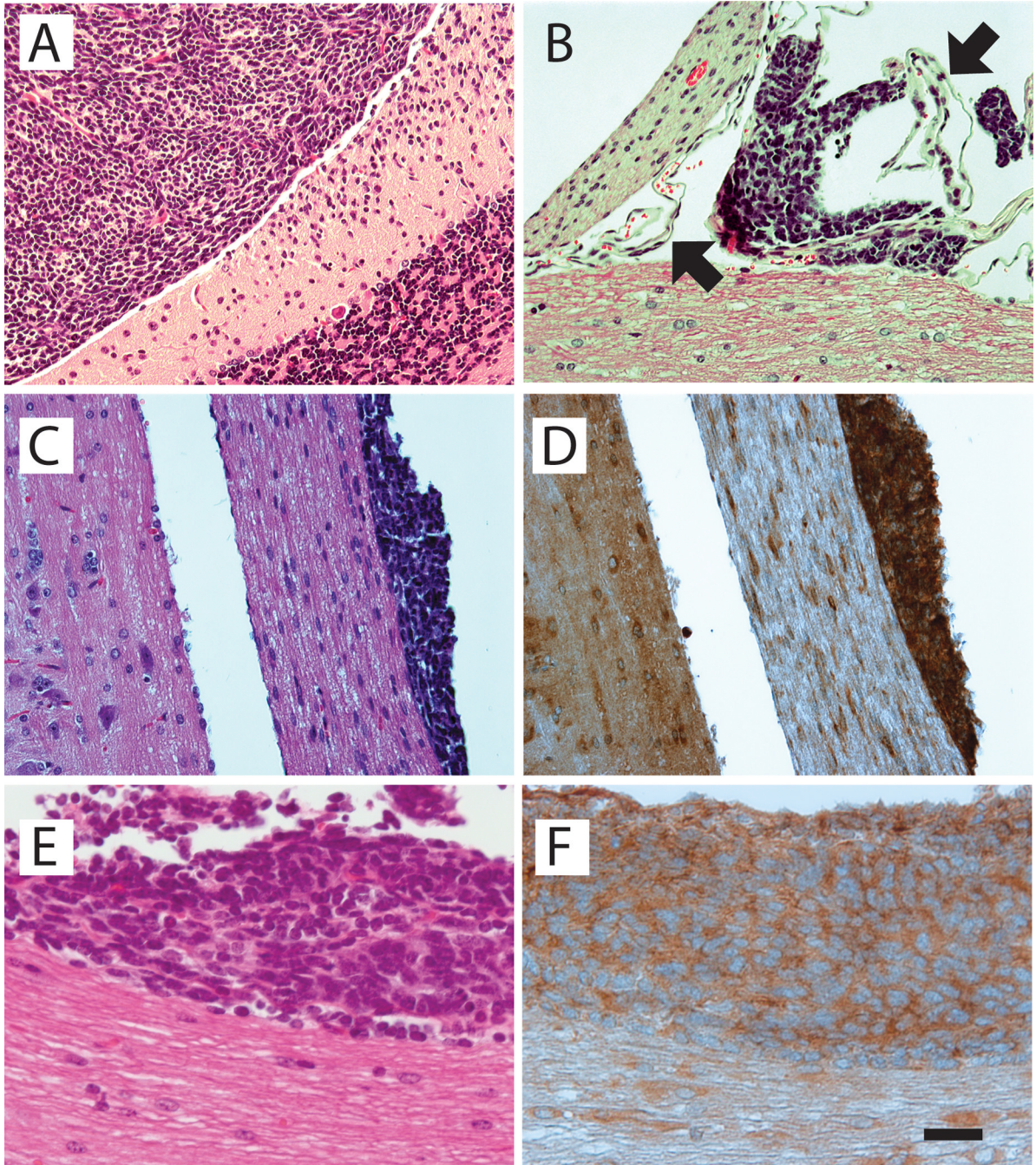
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**Figure 1.**

The Sleeping Beauty transposon system. SB consists of two components, the transposable element (transposon) and the transposase enzyme, which catalyzes transposon mobilization. *A*, Vector for expressing the transposase under control of a tissue-specific promoter/enhancer. *B*, The SB transposon contains a pair of inverted repeat/direct repeat elements (IRDR), flanking the mobile cargo sequence. For cancer gene identification, the cargo sequence is designed to mimic retroviral insertional mutagenesis. The transposon contains splice acceptor sites (SA) and polyadenylation (pA) sequences to disrupt the expression of genes into which the transposon integrates (mRNA1). The transposon also contains 5' sequences from the murine stem cell virus (MSCV) long terminal repeat (LTR) to serve as promoter/enhancer elements, which increase expression of adjacent genes (mRNA2). The MSCV LTR is followed by a splice donor (SD). Thus, a transcript initiated in the LTR can splice into downstream exons of endogenous genes. The SB transposon schematized here is the T2/Onc vector [reviewed in (16)].



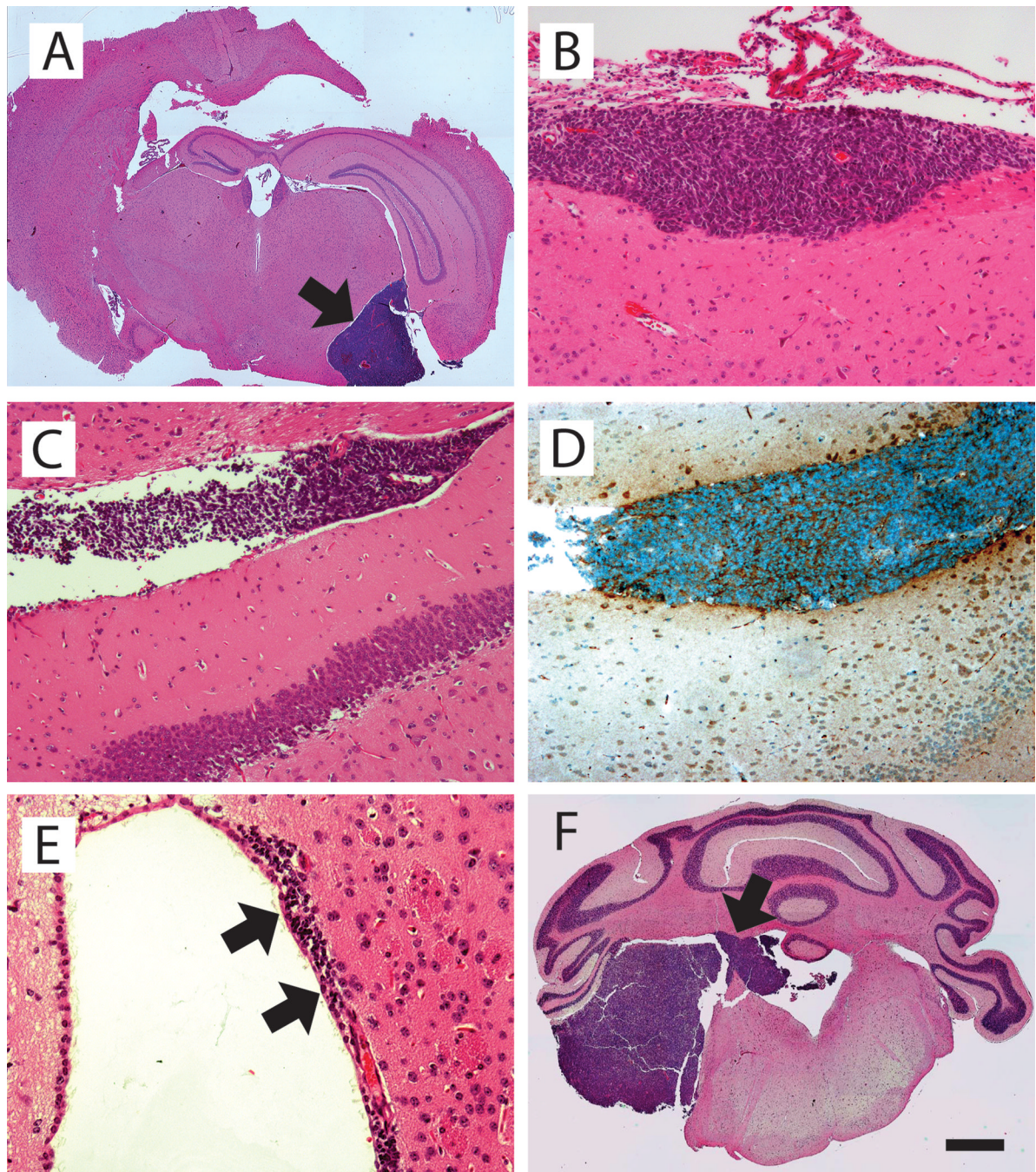


**Figure 2.**

Histopathology of spinal leptomeningeal dissemination. *A*, classic cytoarchitecture of Shh-induced medulloblastoma, showing a uniform field of undifferentiated tumor cells (left) abutting the cerebellar cortex (right) (H&E). *B*, aggregates of Shh-induced medulloblastoma cells attached to the leptomeninges (arrows) of the spinal cord (below) and a spinal nerve (left) (H&E). *C*, metastasis of Shh+Lhx1-induced medulloblastoma cells to a spinal nerve (right) exiting the spinal cord (left) (H&E). *D*, immunoperoxidase staining of tissue section adjacent to that shown in *C*, showing GFP immunoreactivity in tumor cells, thus verifying expression of retrovirus-transferred Lhx1. *E*, metastasis of Shh+Ccrk-induced medulloblastoma to the surface of the spinal cord (H&E). *F*, tissue section adjacent to that

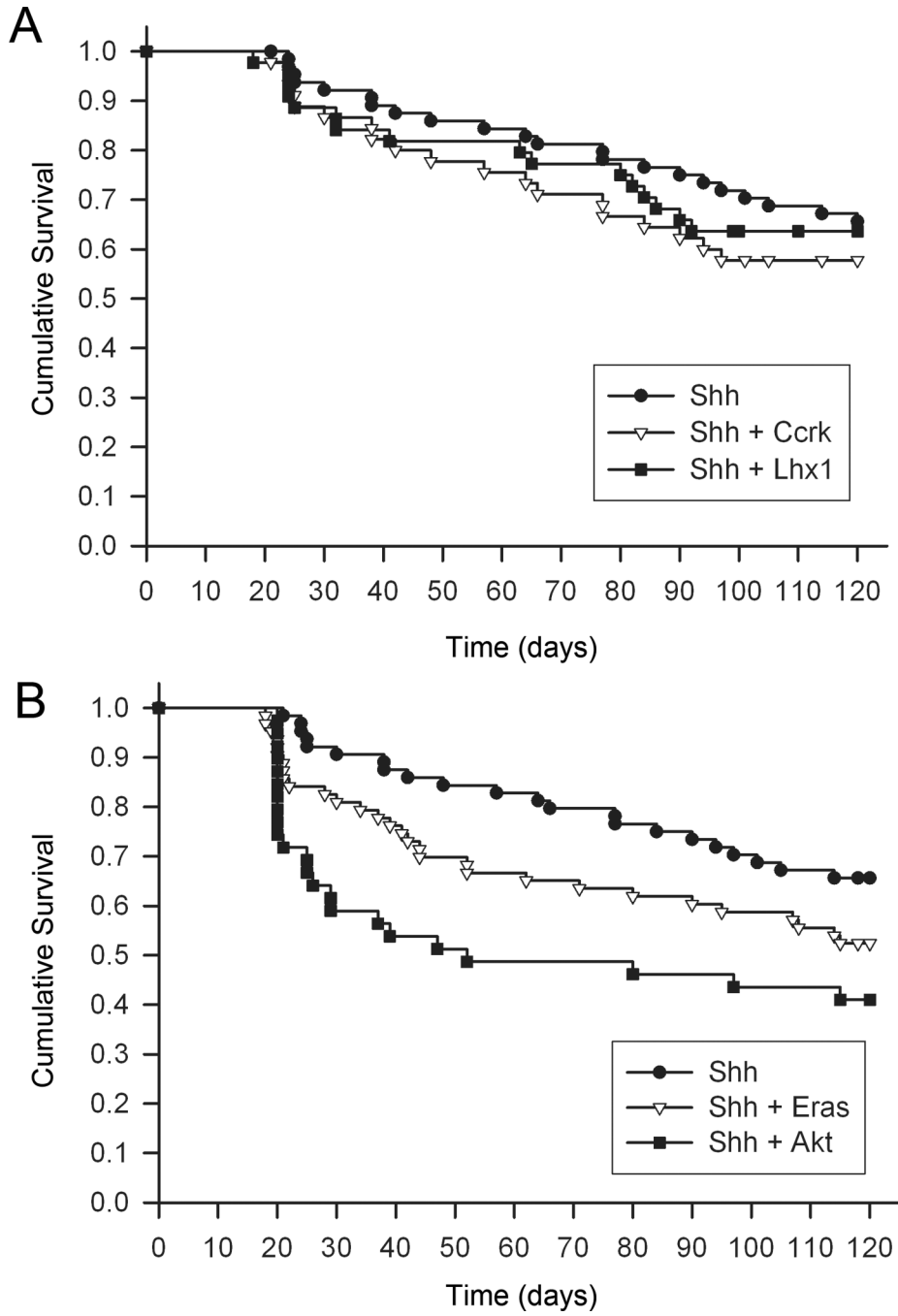
shown in *E*, showing GFP immunoreactive staining in Shh+Ccrk-induced medulloblastoma cells attached to the spinal cord. Scale bar, 50  $\mu\text{m}$  (*A–D*), 25  $\mu\text{m}$  (*E–F*).



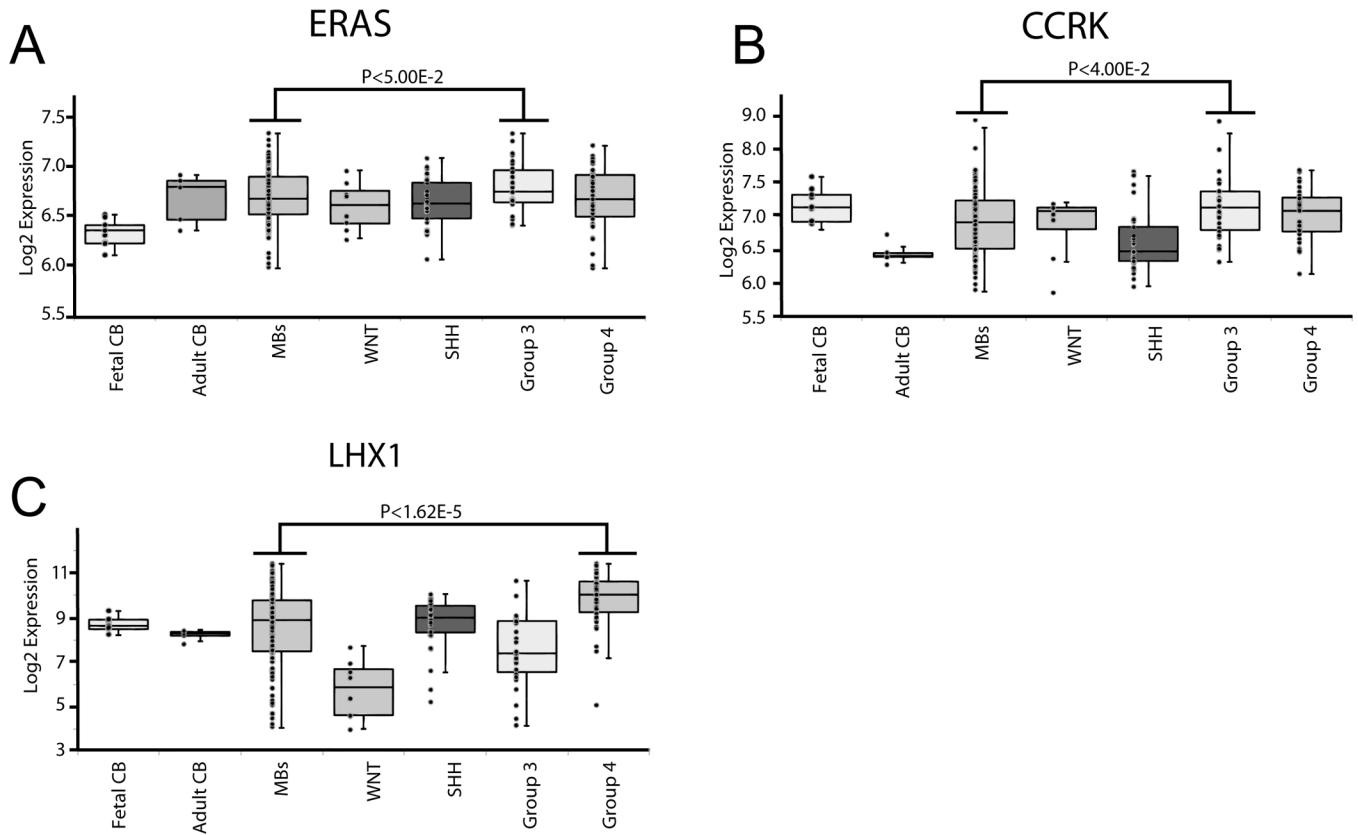


**Figure 3.**

Histopathology of forebrain leptomeningeal dissemination. *A*, infiltration of Shh+Ccrk–induced medulloblastoma (arrow) into the brain stem and hippocampal fissure (H&E). *B*, Shh+Lhx1–induced medulloblastoma cells attached to the leptomeninges of the brain stem (H&E). *C*, Shh+Lhx1–induced medulloblastoma cells in the hippocampal fissure. Granule neurons of the dentate gyrus are visible below (H&E). *D*, GFP immunoreactive staining in metastasizing cells from Shh+Lhx1–induced medulloblastoma. Tissue section is adjacent to that shown in *C*. *E*, Shh+Ccrk–induced medulloblastoma cells in the subependymal space of the lateral ventricle (arrows) (H&E). *F*, Shh-induced medulloblastoma infiltrating the fourth ventricle (H&E). Scale bar, 500  $\mu$ m (*A*, *F*), 100  $\mu$ m (*B–D*), 50  $\mu$ m (*E*).



**Figure 4.** Survival analysis of mice following retroviral transfer of Shh and metastasis-inducing oncogenes. Kaplan-Meier survival analysis of mice injected with RCAS-Shh, alone or in combination with RCAS-Ccrk and RCAS-Lhx1 (A) or RCAS-Eras and RCAS-Akt (B). The mice were injected on day zero and sacrificed at the indicated time points.

**Figure 5.**

Expression of gCIS-associated genes in human medulloblastoma subgroups. Box plots showing relative expression of *ERAS* (A), *CCRK* (B), and *LHX1* (C) in normal cerebella (CB; fetal n=9, adult n=5) and medulloblastoma samples (MB; n=103) profiled on Affymetrix exon arrays. The 103 medulloblastomas were divided into subgroups (WNT, SHH, Group 3, Group 4) and analyzed separately. Log2 expression is a measure of the luminosity of the gene probe signal, corrected for the background luminosity of each array and normalized using control probes across different arrays.



**Table 1**

Incidence of medulloblastoma formation in brain and spine leptomeningeal space during a 4-month observation period

| Genes transferred | Brain tumor incidence | Spine tumor incidence* | P value <sup>†</sup> (spine) |
|-------------------|-----------------------|------------------------|------------------------------|
| <i>Shh</i>        | 27 of 64 (42%)        | 3 of 27 (11%)          | –                            |
| <i>Shh + Eras</i> | 34 of 63 (54%)        | 10 of 34 (29%)         | 0.083                        |
| <i>Shh + Lhx1</i> | 24 of 44 (55%)        | 8 of 24 (33%)          | 0.054                        |
| <i>Shh + Ccrk</i> | 23 of 45 (51%)        | 10 of 23 (43%)         | 0.007                        |
| <i>Shh + Akt</i>  | 22 of 39 (56%)        | 9 of 22 (41%)          | 0.016                        |

\* Spine tumor incidence calculated as a percentage of mice with histologically verified brain tumors.

<sup>†</sup> P values were calculated using  $\chi^2$  contingency test to compare spine tumor incidence after combined gene transfer versus transfer of *Shh* alone.