Tumor and Stem Cell Biology

Identification of FoxR2 as an Oncogene in Medulloblastoma

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Abstract

Medulloblastoma is the most common pediatric brain tumor, and in ~25% of cases, it is driven by aberrant activation of the Sonic Hedgehog (SHH) pathway in granule neuron precursor (GNP) cells. In this study, we identified novel medulloblastoma driver genes through a transposon mutagenesis screen in the developing brain of wild-type and Trp53 mutant mice. Twenty-six candidates were identified along with established driver genes such as Gli1 and Crebbp. The transcription factor FoxR2, the most frequent gene identified in the screen, is overexpressed in a small subset of human medulloblastoma of the SHH subtype. Tgf2 and Alx4, 2 new putative oncogenes identified in the screen, are strongly expressed in the SHH subtype of human medulloblastoma. Mutations in these two genes were mutually exclusive with mutations in Gli1 and tended to cooccur, consistent with involvement in the SHH pathway. Notably, Foxr2, Tgf2, and Alx4 activated Gli-binding sites in cooperation with Gli1, strengthening evidence that they function in SHH signaling. In support of an oncogenic function, Foxr2 overexpression transformed NIH3T3 cells and promoted proliferation of GNPs, the latter of which was also observed for Tgf2 and Alx4. These findings offer forward genetic and functional evidence associating Foxr2, Tgf2, and Alx4 with SHH subtype medulloblastoma. Cancer Res; 74(8); 1–11. ©2014 AACR.

Introduction

Medulloblastoma is the most common malignant brain tumor of childhood and tends to metastasize throughout the central nervous system. Despite overall improvements in survival with multimodal treatment, a substantial proportion of patients are still incurable. Moreover, survivors often suffer considerable treatment-related morbidities, such as neurocognitive deficits related to radiation therapy. To develop better therapies, insights into the molecular mechanisms leading to the development of medulloblastomas are essential. Recent molecular studies have shown that medulloblastomas are composed of at least four distinct subtypes: Sonic Hedgehog (SHH), WNT, Group 3, and Group 4 (1). Tumors characterized by aberrant activation of the SHH pathway (SHH subtype) originate from cerebellar granule neuron precursor (GNP), whereas tumors with activating mutations in the WNT effector CTNNB1 (WNT subtype) arise outside the cerebellum from cells of the dorsal brainstem (2). Although mutations in the SHH and WNT pathway seem mutually exclusive, these alterations affect only 30% to 40% of medulloblastomas (3), suggesting that other pathways are also operative in medulloblastoma formation. To better understand the pathogenesis of medulloblastomas, a comprehensive understanding of genes and pathways that regulate tumor formation in the cerebellum is essential.

Genome-wide insertional mutagenesis is an unbiased and high-throughput method to profile the landscape of driver genes in a mouse model system (4). The Sleeping Beauty (SB) transposon system has expanded the applicability of insertional mutagenesis for the study of various types of solid tumors (5). To identify genes and pathways that can transform embryonic neural stem and progenitor cells to brain tumor-initiating cells, we applied SB transposon mutagenesis to the developing mouse brain. A previous SB mutagenesis study screened medulloblastoma candidate genes by analyzing recurrent insertions in medulloblastomas that developed on Pten heterozygous or Trp53 (+/− and −/−) mouse mutant genetic backgrounds (6) and identified genes that promote metastatic dissemination of medulloblastomas (7). In contrast, we used wild-type or Trp53 mutant (R172H/+) genetic backgrounds that do not develop medulloblastomas spontaneously. Transposition of the mutagenic SB transposons induced medulloblastomas in the cerebellum even on the wild-type background. Here, we describe the identification and functional analyses of candidate genes that may regulate the initiation and/or progression of medulloblastoma.

Materials and Methods

Mouse strains

Mouse strains used in this study are described previously (8). All manipulations were performed with institutional animal
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care and use committee approval (Institute of Molecular and Cell Biology).

Cell culture

Mouse embryonic fibroblasts (MEF) were generated from ICR mouse embryos at embryonic day 13.5. Primary cerebellar cultures were prepared from the cerebellum of ICR mice at postnatal day 5 as described (9). Briefly, the cerebellum was treated with 0.25% trypsin with 0.1% DNaseI (Invitrogen), and then dissociated by trituration. The dissociated cells were cultured in DMEM/F12 medium supplemented with 10% FBS and penicillin/streptomycin. MEFs, cerebellar cells, and NIH3T3 cells (American Type Culture Collection, ATCC) were infected with retrovirus and selected for puromycin resistance. For cotransduction experiments, doubly infected cells were selected with puromycin and G418. Foci formation assay and colony formation assay (more than 50 colonies were counted in three samples), or to 8-well chamber slide for immunostaining (more than 100 Math1-positive cells were counted in three samples).

Immunohistochemical analysis

Immunohistochemistry was performed as described (8). Each tumor was obtained from a different mouse. Anti-Math1 antibody is a generous gift from Dr. J. Johnson (University of Texas Southwestern Medical Center, Dallas, TX, ref. 11). The detection of senescence-associated β-galactosidase activity was performed as described (12).

Production of retrovirus stock

Retroviral expression vectors (pMXs-IRES-Puro and pMXs-IRES-Neo) were obtained from Cell Biolabs, Inc. A plasmid containing (more than 100 Math1-positive cells were counted in three samples).

Results

Sleeping Beauty mutagenesis promotes medulloblastoma formation in the cerebellum

To identify novel driver genes for medulloblastoma, we mobilized SB in the brains of wild-type and Trp53R172H mutant mice. Trp53R172H is an inducible lox-stop-lox allele with both dominant-negative and gain-of-function properties (15). Briefly, Nestin-cre (Nes-Cre) transgenic mice (16) were crossed with Trp53R172H mutant mice (15) to generate compound heterozygous Nes-cre/+; Trp53R172H/+ mice. These mice were then crossed to mice homozygous for a transposon concatamer carrying up to 350 copies of the mutagenic transposon T2/Onc2 (17) and an inducible LSL-SB transposase (SBase) targeted to the Rosa26 locus (18) to generate triple (Nes-cre/+; T2/Onc2/+; SBase/+), and quadruple (Trp53R172H/+; Nes-cre/+; T2/Onc2/+; SBase/) mice. Trp53R172H was used as a sensitizing mutation because mutant Trp53 is one of the key drivers of medulloblastoma (19), whereas Nes-cre was used to activate Trp53R172H and SB transposition in neural stem and progenitor cells. Triple transgenic mice showed enlargement of the head at approximately 2 to 4 months of age and a gait disorder, whereas necropsy of these mice revealed tumors within the cerebellum (Fig. 1A–C). In contrast, control animals (T2/Onc2/+; SBase+) remained tumor free (Fig. 1A, black line). Histoxylin and eosin (H&E) staining of tumors showed classical features of medulloblastoma, including small round tumors composed of sheets of undifferentiated cells with minimal cytoplasm (Fig. 1D and E). Tumors were negative for glioma features of medulloblastoma, including small round tumors composed of sheets of undifferentiated cells with minimal cytoplasm (Fig. 1D and E). Tumors were negative for glioma.

Sequence analysis of SB insertion sites in medulloblastomas

To identify driver genes for medulloblastoma, we PCR-amplified and sequenced the transposon insertion sites from 17 wild-type and 27 Trp53R172H mutant tumors (Table 1). In total, 7,806 and 13,057 nonduplicated SB insertions were of skin cell origin (15). Common insertion sites (CIS) were identified in described (8). To analyze fusion transcripts, cDNA synthesis was performed using RevertraAce (Toyobo) and subsequent PCR was performed using the primers listed in Supplementary Table S1. PCR products were TA-cloned into pGEM-Teasy (Promega) and sequenced. Real-time PCR analysis was carried out on Light Cycler 1.5 (Roche) using primers in

Supplementary Table S1.
therefore most likely to harbor candidate cancer genes. These CISs were then annotated to the nearest mouse gene (CIS genes; ref. 20). Thirteen CIS genes were identified in wild-type tumors (Fig. 2A) and 18 CIS genes were identified in Trp53R172H mutant tumors (Fig. 2B). Five CIS genes, including Foxr2, Tgif2, Alx4, Crebbp, and Wac, were identified in both wild-type and Trp53R172H mutant tumors (Fig. 3A), indicating that these genes regulate medulloblastoma formation irrespective of the genetic background.

To determine whether the CIS genes are enriched in signaling pathways important in cancer, we made use of the DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/). A Gene Ontology term enrichment analysis of the combined CIS gene list (26 genes in Fig. 3A) identified processes associated with transcriptional regulation as being highly significant (Fig. 2C). Ten out of 26 CIS genes (38%) are involved in transcription, and include transcription factors (Foxr2, Tgif2, Alx4, Gli1, and Hivep3), transcription cofactors (Crebbp and Trim33), a histone acetyltransferase (Crebbp), a histone methyltransferase (Whsc1l1), a regulator of histone H2B ubiquitination (Wac), and a component of the SWI/SNF chromatin remodeling complex (Arid1b).

### Table 1. Analysis of transposon insertions in medulloblastomas

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mice necropsya</th>
<th>Medulloblastomasb</th>
<th>Tumor samplesc</th>
<th>Number of insertionsd</th>
<th>Number of CIS genes</th>
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</thead>
<tbody>
<tr>
<td>T2/Onc2/+; SBase/+ (n = 5)</td>
<td>5</td>
<td>0 (0%)</td>
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<td></td>
<td></td>
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<tr>
<td>Nes-cre/+; T2/Onc2/+; SBase/+ (n = 43)</td>
<td>19</td>
<td>17 (40%)</td>
<td>17</td>
<td>7,806</td>
<td>13</td>
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<tr>
<td>Trp53R172H/+; Nes-cre/+ (n = 10)</td>
<td>10</td>
<td>0 (0%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp53R172H/+; Nes-cre/+; T2/Onc2/+; SBase/+ (n = 58)</td>
<td>37</td>
<td>34 (59%)</td>
<td>27</td>
<td>13,057</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>20,863</td>
<td>26</td>
<td></td>
<td></td>
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</table>

*The number of mice that were sent to necropsy.

bThe number of mice with medulloblastoma at histologic analysis.

CThe number of tumor samples used for the analysis of transposon insertions. Each tumor is from a different mouse.

dInsertions in the transposon donor chromosomes were not included because of problem caused by local hopping.
**Figure 2.** CIS genes identified in medulloblastomas. A, thirteen CIS genes were identified in wild-type tumors. B, eighteen CIS genes were identified in Trp53 mutant tumors. *P* values were calculated as described (20). Prediction on gene function indicates whether the T2/Onc2 insertion would cause transcriptional activation of a gene (drives) or would disrupt gene transcription (disrupts) on the basis of the position and orientation of the T2/Onc2 insertion relative to gene transcription. C, GO analysis of biological processes of CIS genes in medulloblastomas.

**Table 3.** WT tumors and Trp53-mutant tumors.

**A** WT tumors

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of insertions</th>
<th>%Tumors (n=17)</th>
<th>Predicted effect</th>
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<tbody>
<tr>
<td>Foxr2</td>
<td>7</td>
<td>&lt;1.0E-10</td>
<td>35%</td>
</tr>
<tr>
<td>Wac</td>
<td>6</td>
<td>2.6E-06</td>
<td>24%</td>
</tr>
<tr>
<td>Alx4</td>
<td>5</td>
<td>1.3E-06</td>
<td>29%</td>
</tr>
<tr>
<td>Tgf2</td>
<td>9</td>
<td>3.6E-06</td>
<td>22%</td>
</tr>
<tr>
<td>Mag3</td>
<td>14</td>
<td>1.3E-05</td>
<td>29%</td>
</tr>
<tr>
<td>Cnkr2</td>
<td>7</td>
<td>2.0E-05</td>
<td>24%</td>
</tr>
<tr>
<td>Pipid</td>
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<td>24%</td>
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<tr>
<td>Hhgl2</td>
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<tr>
<td>Pde5a</td>
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<td>Kalm</td>
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<td>41%</td>
</tr>
<tr>
<td>Cebbp</td>
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<tr>
<td>Vps13b</td>
<td>8</td>
<td>2.4E-04</td>
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**B** Trp53-mutant tumors

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of insertions</th>
<th>%Tumors (n=27)</th>
<th>Predicted effect</th>
</tr>
</thead>
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<tr>
<td>Foxr2</td>
<td>15</td>
<td>&lt;1.0E-10</td>
<td>44%</td>
</tr>
<tr>
<td>Tgf2</td>
<td>12</td>
<td>&lt;1.0E-10</td>
<td>33%</td>
</tr>
<tr>
<td>Crebbp</td>
<td>9</td>
<td>&lt;1.0E-10</td>
<td>26%</td>
</tr>
<tr>
<td>Hlvep3</td>
<td>7</td>
<td>&lt;1.0E-10</td>
<td>22%</td>
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<td>Dusp8</td>
<td>7</td>
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<td>As30006K03Rik</td>
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<td>Trim33</td>
<td>7</td>
<td>5.9E-06</td>
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<td>Wac</td>
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<tr>
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<td>6</td>
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<td>7</td>
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<td>Akap6</td>
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<td>7</td>
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<td>22%</td>
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<tr>
<td>C330045G13Rik</td>
<td>5</td>
<td>3.3E-05</td>
<td>7%</td>
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<tr>
<td>Gli1</td>
<td>5</td>
<td>3.9E-05</td>
<td>19%</td>
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<tr>
<td>Alx4</td>
<td>7</td>
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<td>22%</td>
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<tr>
<td>Arid1b</td>
<td>13</td>
<td>4.7E-05</td>
<td>37%</td>
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<tr>
<td>Atxn1</td>
<td>14</td>
<td>1.2E-04</td>
<td>37%</td>
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<tr>
<td>Whsc1l1</td>
<td>12</td>
<td>1.7E-04</td>
<td>33%</td>
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**C** Regulation of transcription

<table>
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<th>Value</th>
<th>Regulation of transcription</th>
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<tr>
<td>0</td>
<td>Negative regulation of gene expression</td>
</tr>
<tr>
<td>1</td>
<td>Positive regulation of gene expression</td>
</tr>
<tr>
<td>2</td>
<td>Positive regulation of transcription</td>
</tr>
</tbody>
</table>

**GLI1,** a downstream effector of SHH signaling, is often amplified in medulloblastomas of the SHH subtype (21). GLI1 was identified as a CIS gene in Trp53 mutant tumors (Fig. 3A). All GLI1 insertions were located upstream of exon 6, in the same transcriptional orientation as GLI1 (Fig. 3B), suggesting that SB might be upregulating GLI1 expression from the MSCV promoter contained within the transposon, consistent with its known role as an oncogene. To confirm this, we PCR-amplified SB–GLI1 fusion transcripts with primers that recognize the splice donor site located downstream of the MSCV promoter and the coding region of GLI1. Sequencing of the amplified transcripts showed that the MSCV promoter was spliced to exon 6 of GLI1 in both tumors examined with GLI1 insertions (Fig. 3I), resulting in the overexpression of a truncated transcript. Sequencing of the fusion transcripts showed that the MSCV promoter was spliced to exon 2 (Fig. 3J), again driving the expression of a full-length transcript. Tgf2 is a member of the TALE homeodomain family. Alx4 belongs to the family of aristaless-like homeobox genes. Alx4 insertions are similarly sense-oriented and located upstream of the gene or within intron 1 (Fig. 3E). Sequencing of the fusion transcripts showed that the MSCV promoter was spliced to Alx4 exon 2 (Fig. 3K), inducing the expression of a truncated protein that may initiate from an ATG codon located in exon 2.

In contrast, Crebbp and Wac insertions were mostly distributed throughout the genes, and there was little orientation bias (Fig. 3F and G). The average numbers of Crebbp and Wac insertions in tumors are 23 and 13 respectively, suggesting a haploinsufficient role for Crebbp and Wac in tumor suppression. Crebbp functions as a transcriptional coactivator, and haploinsufficiency of CREBBP is responsible for Rubinstein–Taybi syndrome, an autosomal congenital disorder characterized by developmental and pathologic phenotypes, including predisposition to tumors including medulloblastoma (24, 25). Somatic heterozygous nonsense Crebbp mutations have been identified in medulloblastomas (26). WAC is a functional partner of histone H2B ubiquitin ligase RNF20/40, and the depletion of RNF20/40 or WAC abolishes H2B monoubiquitination (27). Although significant loss of RNF20/40 is catastrophic to cells, more subtle changes in RNF20/40 expression because of partial knockdown allow cells to proliferate and manifest dramatic genomic instability (28). These data support a haploinsufficient role for Crebbp and Wac in medulloblastomas.

A previous transposon screen that analyzed for recurrent insertions in medulloblastomas, which developed on a Pch1 heterozygous genetic background (6) did not identify GLI1, Foxr2, Tgf2, or Alx4, raising the possibility that these genes have overlapping function with Pch1 deficiency. We also...
looked for co-occurring and mutually exclusive mutations in pairs of CIS genes. A significant co-occurrence of mutations in Tgf2 and Alx4 insertions was observed (Fig. 3M and N; P = 0.042 after multiple testing correction), whereas Tgf2 and Alx4 insertions were mutually exclusive to Gli1 insertions (Fig. 3M).

Expression of GLI1, FOXR2, TGF2, and ALX4 in human medulloblastomas

Because the patterns of transposon insertions predict oncogenic functions for GLI1, FOXR2, TGF2, and ALX4 in medulloblastoma, we next determined whether expression of these genes was upregulated in human medulloblastoma using a microarray dataset for 285 medulloblastomas (29) that was accessible through the open access database R2 (http://r2.amc.nl). Not surprisingly, GLI1 is strongly expressed in medulloblastomas of the SHH subtype (Supplementary Fig. S1A). Interestingly, most tumors that strongly express TGF2 also belong to the SHH subgroup (Supplementary Fig. S1B). ALX4 and FOXR2 are also strongly expressed in medulloblastoma, but in more limited subsets of tumors (Supplementary Fig. S1C and S1D), and once again most of these tumors are of the SHH subgroup. We also found that most of the tumors highly expressing ALX4 also expressed TGF2 at high levels (Supplementary Fig. S1E), showing significant co-occurrence of ALX4 and TGF2 in medulloblastomas (P < 0.001, Fisher exact test). Taken together, these data indicate that these 4 putative oncogenes are overexpressed in a subset of human medulloblastomas that belong to the SHH subgroup.

Foxr2, Tgf2, and Alx4 enhance transcriptional activity of Gli1

Because FOXR2, TGF2, and ALX4 are expressed in a subset of SHH subgroup medulloblastomas, we next tested whether these genes can activate a SHH responsive element, the Gli-binding site (Gli-BS; ref. 30). Transfection of a vector expressing full-length Gli1 increased reporter expression in HEK-293T cells; however, expression of the reporter with mutated Gli-binding...
site (mGli-BS; ref. 30) was not changed (Fig. 4A). Transfection of vectors expressing Foxr2, Tgif2, or Alx4 alone did not affect reporter expression; however, cotransfection of these vectors along with the Gli1 expression vector increased Gli1 reporter expression above that seen with the Gli1 expression vector alone (Fig. 4A and B). This upregulation of Gli1-dependent reporter expression was upregulated by cotransfection with Foxr2, Alx4, or Tgif2 expression vectors. Data represent means ± SEM with three samples per group. The amounts of DNA (μg) used for transfection are shown. *, P < 0.05 and **, P < 0.01 (Student t test, one-tailed). B, the experiments shown in A were repeated three times, and data represent means ± SEM with 9 samples per group. ***, P < 0.01 (Student t test, one-tailed). C, dose-dependent cooperative effects were observed for Foxr2, Alx4, and Tgif2. Data represent means ± SEM with 9 samples per group. **, P < 0.01 (Student t test, one-tailed). D, the expression of Ptc1 and Ptc2 in NIH3T3 cells overexpressing Foxr2, Alx4, or Tgif2. Expression was normalized using β-actin as an internal control. Data represent means ± SEM with three samples per group. *, P < 0.05 and **, P < 0.01 (Student t test, one-tailed). E, cooperation between Alx4 and Tgif2 on activation of Gli-dependent transcription. Data represent means ± SEM with 9 samples per group. **, P < 0.01 (Student t test, one-tailed).
expression was observed dose dependently (Fig. 4C). As expected, expression of endogenous SHH target genes in NIH3T3 cells, Ptc1 and Ptc2 (31) were upregulated by Foxr2, Tgif2, or Alx4 (Fig. 4D). Cotransfection of Tgif2 and Alx4 induced stronger luciferase reporter expression compared with Tgif2 or Alx4 alone (Fig. 4E), providing evidence for functional cooperation between these genes. Taken together, these results demonstrate cooperative functions of these genes (Foxr2, Tgif2, or Alx4) and Gli1 in Gli-dependent transcriptional activation.

**Functional analysis of candidate medulloblastoma driver genes**

To examine the oncogenic functions of candidate genes, we first measured their transforming activity in NIH3T3 cells. We infected NIH3T3 cells with retroviruses expressing full-length Foxr2, Tgf2, truncated Alx4, or an empty vector. Foxr2 overexpression induced morphologic changes (Fig. 5C), and produced a significantly greater number of NIH3T3 foci, whereas truncated Alx4 did not promote and Tgf2 suppressed foci formation (Fig. 5D). Analysis of cell growth in low-serum media also showed that Foxr2-expressing cells grew more efficiently (Fig. 5E). These data indicate transforming activity of Foxr2 in NIH3T3 cells.

We next analyzed the effects of Foxr2 overexpression on MEFs. Normal MEFs undergo senescence as a result of serial passage in culture (32). As expected, control MEFs became enlarged and ceased to proliferate after several passages, and these cells expressed senescence-associated β-galactosidase (Fig. 5F and G). However, Foxr2-expressing MEFs retained their
small cell shape and continued to proliferate (Fig. 5F and G), and the proportion of β-galactosidase expressing cells decreased (Fig. 5H). p16Ink4a, a cyclin-dependent kinase inhibitor, is also expressed in most senescent cells (32). As expected, the expression level of p16Ink4a increased during passages of MEFs; however, its expression was significantly lower in MEFs overexpressing Foxr2 (Fig. 5I). Taken together, these results indicate that overexpression of Foxr2 prevents MEFs from entering senescence.

Cerebellar GNPs are considered to be the cell-of-origin of the SHH subgroup of medulloblastomas (3). We therefore next examined the effects of overexpression of Foxr2, truncated Alx4, and Tgif2 on cultures of primary cerebellar cells. These cultures consisted primarily of GNPs and astrocytes (9). At 1 week after plating, colonies consisting of GNPs were distinguishable from surrounding large flat astrocytes because of their small cell shape and cytoplasmic granules (Fig. 6A–C). These small granular cells expressed Math1, a specific marker for GNPs.

Figure 6. Overexpression of medulloblastoma candidate genes promotes proliferation of GNPs. A, cerebellar cells infected with retroviruses expressing Foxr2, Alx4, Tgif2, or empty vector. Arrowheads, large colonies consisting of GNPs. Bars, 250 μm. B, magnified views of GNP colonies. Bars, 50 μm. C, GNP colonies contained cytoplasmic granules that were visible under differential interference contrast (DIC) microscopy (left). These granular cells expressed Math1, but were negative for S100b (right). Arrow, the nucleus of an astrocyte. Nuclei were counterstained with DAPI. Bars, 50 μm. D–F, colony size and proliferation of GNPs were compared among control, Foxr2, Alx4, and Tgif2-expressing GNPs. Foxr2-expressing GNPs generated larger colonies than control (D; Mann–Whitney test, P < 0.001). The fraction of Ki67-expressing Math1-positive GNPs was increased by Foxr2 overexpression (E and F). Data represent means ± SEM with three samples per group. **, P < 0.01 (Student t test, one-tailed). Data are representative of two independent experiments. G, cerebellar cells infected with retroviruses expressing Alx4 and Tgif2, or Alx4 and empty vector. Most of the control GNPs remained single (arrows), whereas cotransduction of Alx4 and Tgif2 induced colonies consisting of GNPs (arrowheads). Bars, 250 μm (top) and 50 μm (bottom). H and I, GNPs transduced with Alx4 and Tgif2 generated larger colonies than control (Mann–Whitney test, P < 0.001; H). The fraction of Ki67-expressing Math1-positive GNPs was increased by cotransduction of Alx4 and Tgif2 (I). Data represent means ± SEM with three samples per group. **, P < 0.01 (Student t test, one-tailed). Data are representative of two independent experiments.
for GNPs (9, 11), but were negative for an astrocyte marker, S100b (Fig. 6C). The colony size was larger in Foxr2-expressing GNPs than control cells (Fig. 6A, B, and D). The expression of Ki67, a marker associated with cellular proliferation, was also increased by Foxr2 overexpression (Fig. 6E and F). These findings demonstrate a growth promoting function for Foxr2 in primary GNPs. Although Alx4 or Tgf2 alone did not promote proliferation (Fig. 6D and F), cotransduction of Alx4 and Tgf2 induced colonies with larger sizes than Alx4 alone (Fig. 6G and H). Ki67 expression was also increased by cotransduction of Alx4 and Tgf2 (Fig. 6I), providing evidence for cooperation between these genes in GNPs.

Discussion

Transposon-based insertional mutagenesis allows for unbiased, whole genome screens for cancer genes, and has been used successfully to model many different types of cancer in mice (5). In this study, we used transposon mutagenesis to model medulloblastoma. Using Nes-cre transgenic mice, we mobilized T2/Onc2 transposons in neural stem and progenitor cells of the developing brain. As a result, mutagenic transposons induced medulloblastomas in the cerebellum. Strikingly, we observed medulloblastomas even on a wild-type genetic background. These tumors are invaluable because they have the potential to identify driver genes that can initiate tumorigenesis rather than just accelerate tumorigenesis induced by mutation of another gene, such as a mutation of Ptch1. Subsequent sequence analysis of the transposon integrations sites in these tumors identified known medulloblastoma-associated genes Gli1 and Crebbp as well as genes that have not been associated with medulloblastoma, including Foxr2, Tgf2, Alx4, and Wac.

A previously published transposon screen identified medulloblastoma candidate genes by analyzing for recurrent insertions in medulloblastomas that developed on heterozygous Ptch1 mutant or Trp53 mutant (+/− and −/−) backgrounds (6). Ptch1 is a negative regulator of the SHH signaling pathway (33) and not surprisingly, Ptch1 heterozygous mice spontaneously develop medulloblastomas (34). CIS genes identified on the Ptch1 mutant background were shown to promote metastatic dissemination of medulloblastoma (7). The previous study (6) also identified Ptch1 in medulloblastomas induced on Trp53 mutant backgrounds. The identification of Ptch1 on the Trp53 −/− background is consistent with a previous report describing cooperation between Trp53 loss and Ptch1 heterozygosity for medulloblastoma formation (35). In contrast, it has been reported that Ptch1 heterozygosity does not accelerate medulloblastoma formation on Trp53 heterozygous background (35). This may explain why Ptch1 was not identified in our screen on the Trp53+/− heterozygous mutant background. We also used a wild-type background, which did not develop medulloblastoma spontaneously. Therefore, some of the CIS genes identified in our screen are likely to represent driver genes involved in the initiation of medulloblastoma. Comparison of our CIS genes with those identified in the Ptch1 and Trp53 mutant screen showed that 5 genes were identified in both screens, including Aريد1b, Crebbp, Trim33, Map2k4, and Wac, providing further evidence that these genes are medulloblastoma disease genes. The patterns of transposon insertions predict tumor suppressor functions for these genes. In fact, recurrent mutations in cancers have been reported for TRIM33 (36), Aريد1b (37), and Map2k4 (38). Interestingly, genes such as Gli1, Foxr2, Tgf2, and Alx4 were not identified in the Ptch1 and Trp53 mutant screen (7), raising the possibility that these genes might function in SHH signaling and were therefore not identified because the SHH pathway was already activated by either Ptch1 germline or transposon-induced somatic mutations.

The most frequently mutated gene identified in our screen was Foxr2. Fox proteins can both activate and repress gene expression through the recruitment of cofactors or repressors, primarily histone deacetylases. Derepression of Fox proteins is commonly associated with cancer progression (39). FOXR2 has high homology to FOXRI, which can functionally replace MYC and drive proliferation of a neural crest cell line (40). A recent mutagenesis screen identified Foxr2 as a proto-oncogene in malignant peripheral nerve sheath tumors (41). This study provides the first forward genetic evidence associating Foxr2 with medulloblastoma. Foxr2 insertion pattern predicts an oncogenic role for Foxr2. Consistent with this, Foxr2 overexpression showed transforming activity in NIH3T3 cells, suppressed senescence in primary fibroblasts and promoted the proliferation of GNPs. Interestingly, Foxr2 was strongly expressed in a small subset of medulloblastomas that belonged to the SHH subgroup of medulloblastoma.

The analysis of co-occurring and mutually exclusive pairs of insertions in CIS genes showed that Gli1 insertions were mutually exclusive to insertions in Tgf2 and Alx4. In addition, we found a significant co-occurrence between insertions in Tgf2 and Alx4. Analysis of the expression of these genes in human medulloblastoma showed that Tgf2 and Alx4 are strongly expressed in a subset of medulloblastomas that belong to the SHH subgroup. This again raises the possibility that Tgf2 and Alx4 functions in SHH signaling. Consistent with this, expression of Gli1 is reduced in Tgf2 conditional null mice, and deficiency of Gli3, a potent repressor of SHH signaling, partially rescues the Tgf2 null phenotype (42). Interestingly, the Alx4 null phenotype can also be partially rescued by loss of Gli3, further suggesting that Alx4 functions in SHH signaling (43). Our studies showing that Tgf2 and Alx4 genes are strongly expressed in the SHH subgroup of medulloblastoma also supports this notion.

Importantly, we found that Foxr2, Tgf2, and Alx4 cooperated with Gli1 and activated Gli-dependent transcription, demonstrating the involvement of these genes in SHH signaling. Tumors with mutations in members of the SHH pathway constitute nearly 25% of medulloblastomas (26, 44) that belong to the SHH subgroup of medulloblastomas, and the aberrant activation of the SHH pathway is considered to be causally related to this subgroup. Although Alx4 and Tgf2 promoted Gli-dependent transcription, these genes did not promote proliferation of GNPs by itself. In contrast, cotransduction of Alx4 and Tgf2 enhanced Gli-dependent transcription and promoted proliferation. A previous study showed that additional factors define an activation threshold for Gli target genes.
(45), raising a possibility that similar mechanisms may regulate a proliferative response to the Gli transcriptional activity in GNPs. The SHH signaling pathway has been implicated in a wide variety of human tumors (46). Analysis of the expression level of FOXR2, TGF2, and ALX4 in human cancer cell lines using the CCLE database (47) revealed that nearly 7% of cancer cell lines showed higher expression of FOXR2 than 1.5 interquartile ranges above the upper quartile (Supplementary Fig. S1F). Interestingly, FOXR2 strongly expressing tumors include cancers associated with SHH signaling such as multiple myeloma, melanoma, and glioma (46), raising a possibility that FOXR2 may also be involved in other cancers associated with SHH signaling. ALX4 and TGF2 were strongly expressed in 2% and 0.1% of cancer cell lines, respectively (Supplementary Fig. S1G and S1H). Although we cannot distinguish the expression of truncated ALX4 from that of full-length ALX4, nearly half of the cell lines that strongly express ALX4 belonged to the lung non–small cell carcinoma, in which SHH signaling plays an essential role (48).

Interestingly, nearly 40% of genes identified in our screen were associated with transcription, consistent with a previous screen for medulloblastoma disease genes (49). Transcription factors play essential roles in coordinated gene expression during development. Recently, the aberration of gene expression has been implicated in mechanisms of cancer formation in the pediatric setting (26, 50), raising the possibility that aberrations of gene expression may perturb the normal developmental program and cause deregulated proliferation of stem or progenitor cells. The finding of transcriptional regulators in our screen supports the notion that deregulation of the developmental program is a core mechanism of pathogenesis in medulloblastomas (3). Taken together, our findings implicate FOXR2, TGF2, and ALX4 as potential therapeutic targets for medulloblastomas as well as for cancers that involve SHH signaling. Future studies will be required to determine the exact roles of these genes in a wide variety of human cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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