OTX2 is critical for the maintenance and progression of classic medulloblastoma

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Abstract

OTX2 is a developmentally regulated transcription factor that plays important roles in early morphogenesis of the central nervous system. We previously identified *OTX2* amplification and overexpression in medulloblastoma cell lines. However, the nature and frequency of *OTX2* genetic alterations have not been determined in large sample sizes of primary tumors. To refine our understanding of genomic events affecting *OTX2*, we analyzed genomic copy number and mRNA expression of OTX2 in a large cohort of primary medulloblastomas. We found that the most frequent focal copy number gain in these primary medulloblastomas (gained in 21% of the tumors, n=201) contained the single gene *OTX2*, indicating that copy number gain of *OTX2* is a driver event in this tumor. In this cohort of tumors, we found that the event was restricted to tumor subtypes that do not express a molecular signature of either Wnt or Shh pathway activation. FISH analysis revealed that *OTX2* copy number gain is present in a subset of cells within medulloblastoma samples, suggesting that it is a late event contributing to tumor progression. Gain of *OTX2* copy number is associated with the presence of anaplastic histological features and shorter survival in medulloblastoma patients. To verify a functional role of the observed genomic events, we demonstrate that ectopic OTX2 expression enhances proliferation and tumorigenicity of immortalized primary cells and that OTX2 knockdown in medulloblastoma xenografts prolongs survival of recipient animals. Finally, we demonstrate that OTX2 transcriptionally upregulates the medulloblastoma oncogene *MYC* as a potential mechanism whereby OTX2 promotes tumor progression.

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Background

Medulloblastoma is the most common malignant brain tumor in children, with a peak incidence at seven years of age (1–3). In recent years, medulloblastoma has been redefined by the development of treatment strategies, including surgical resection, radiation, and chemotherapy. Still, an unacceptably large percentage of children die of this disease, with five-year survival rates reaching only 50–60% (4–6). Moreover, long-term survivors frequently suffer life-long sequelae from aggressive treatment regimens (7–9). More refined tumor grading systems are therefore urgently needed to optimize efficacy and minimize treatment-related toxicity for each patient.

Histologically, medulloblastomas exhibit substantial heterogeneity both among and within tumors, and histological characteristics do not uniformly correlate with clinical prognosis or treatment response. However, molecular analysis of medulloblastomas has shown that incorporating gene expression and genetic alteration data along with clinical and histopathologic data into a patient's profile may better stratify patients into particular treatment and prognostic groups (5,10–13).

Recently, we and others identified *OTX2* amplification and overexpression in medulloblastoma cell lines and primary tumors (14–17). Gene expression analyses revealed that OTX2 transcripts were present at high levels in 14 of 15 (93%) medulloblastomas with anaplastic histopathologic features. Additionally, we demonstrated that knockdown of OTX2 expression by siRNAs inhibited medulloblastoma cell growth *in vitro* (15).

OTX2 is a member of a well-conserved family of bicoid-like homeodomain (HD)-containing transcription factors that play important roles in embryo patterning, brain regionalization, and lineage specification (18). OTX2 is expressed in the prospective cerebellum (the midbrain/hindbrain boundary) of the early embryo as well as in the rapidly expanding population of cerebellar granule cell precursors (19,20). *OTX2*-deficient mice fail to develop the mesencephalon and prosencephalon, and heterozygotes exhibit a lethal "headless" phenotype of variable penetrance (21,22). Although the expression pattern and function of OTX2 in brain morphogenesis have been well-studied in progressive developmental stages of rodent models (20,23), there is little understanding of how OTX2 relates to the clinical and histopathologic features of medulloblastoma or to the molecular pathways of medulloblastoma pathogenesis.

To further our understanding of the genetic mechanism of OTX2 activation and its clinical implications in medulloblastomas, we examined its copy number and expression status in a large cohort of tumors (201 and 103 primary medulloblastomas, respectively). Here we identify recurrent *OTX2* gain (~21%) and, even more commonly, OTX2 overexpression (~74%) in primary medulloblastomas. *OTX2* focal gains were limited to tumors not expressing signatures of Wnt or Shh pathway activation. FISH analysis demonstrated that *OTX2* copy number gain was present in a subset of medulloblastoma cells and was associated with a more aggressive tumor phenotype and adverse clinical outcome. In order to understand its oncogenic functions, we overexpressed OTX2 in immortalized primary cells and demonstrated that OTX2 could promote cell proliferation and tumorigenicity. Furthermore, we determined that stable OTX2 knockdown prolonged survival in a medulloblastoma xenograft model. Finally, we demonstrated that OTX2 transcriptionally regulates the medulloblastoma oncogene *MYC*. These results suggest that OTX2 plays a role in progression and maintenance of a large subset of medulloblastoma tumors and thus represents a promising therapeutic target.

Material and methods

Patients

Medulloblastomas were obtained from the Duke University Medical Center Brain Tumor Biorepository and the Hospital for Sick Children, University of Toronto according to Internal Review Board (IRB) protocol. This biorepository contained specimens obtained from Duke and the (now defunct) Pediatric Oncology Group that had been originally studied for *MYC* amplification (24).

Pathology

Classic, desmoplastic/nodular, and anaplastic/large cell medulloblastomas used in FISH analysis were examined by a neuropathologist (RE McLendon) and classified using the World Health Organization guidelines (1). The classic, or diffuse, pattern of medulloblastoma presents a histologic appearance of densely packed cells with round-to-oval or carrot-shaped hyperchromatic nuclei surrounded by scanty cytoplasm growing in sheets. Neuroblastic, or Homer-Wright, rosettes are most commonly encountered in this group (25).

The anaplastic variant is composed of cells with elongated hyperchromatic nuclei that are densely crowded and exhibit characteristic nuclear wrapping against adjacent tumor cells, abundant individual cell necrosis frequently associated with geographic regions of necrosis, and numerous mitotic figures (26). Moderate anaplasia lacks the degree and extent of necrosis seen in severely anaplastic tumors and is more commonly recognized by a "starry sky" pattern of individual cell necrosis interrupting the sheets of tumor cells.

Single nucleotide polymorphism array and microarray analysis

Hybridization, detection, and analysis of Affymetrix single nucleotide polymorphism (SNP) and exon arrays were carried out as previously described (27,28).

FISH

FISH was performed as described previously (29). BAC clones RP11-1085N6 (56.159277-56.402022 Mb, spanning the *OTX2* gene) and CTD-2505P22 (103.186989-103.414534 Mb, located telomeric to OTX2 on chromosome 14q) were obtained (Invitrogen, Carlsbad, CA) and used to generate probes for the *OTX2* gene and an internal control, respectively. One hundred cells were counted in each of 10 classic medulloblastomas, 11 desmoplastic/nodular medulloblastomas, 10 medulloblastomas with moderate anaplastic features, and 11 medulloblastomas with severe anaplastic features. *OTX2* copy number was determined by the average ratio of green to red probes per cell, and copy number gain was designated as tumors with a ratio greater than 1.2.

Statistical analysis

The significance level for all tests was set a priori at 0.05 by using a 95% confidence interval. All statistical and survival analyses were performed with SAS E-guide software (SAS Institute, Cary, NC).

Plasmid constructs

A full-length cDNA of human *OTX2* was derived from pCMV6-XL5-OTX2 (OriGene, Rockville, MD) and subcloned into the pEGFP-N1 vector.

Cell culture, proliferation, and apoptosis assays

Medulloblastoma and rat kidney epithelium (RK3E) cell lines were maintained as described previously (15,30). Cell line D425MED was derived from a patient with a primary medulloblastoma, whereas D458MED was established independently from cerebrospinal fluid samples of the same patient at a later date. MTT and colony proliferation assays were performed as described (15,30).

Establishing an RK3E line stably expressing OTX2

RK3E cells were transfected with pEGFP-N1 or pEGFP-OTX2. The transfected cells were cultured in DMEM media containing 500 µg/ml G418 (Invitrogen) for 3–4 weeks. Single cell dilution was used to select monoclonal cell lines that were resistant to G418 and expressing OTX2.

Tumorigenicity in athymic nude mice

Groups of seven or eight mice per cell line were used to investigate the tumorigenicity of RK3E cells stably transfected with pEGFP-N1 or pEGFP-OTX2, respectively. We implanted 10^6 cells stereotactically into the cerebral hemisphere of BALB/c athymic nu/nu mice. Brains of euthanized mice were collected, fixed in formalin, paraffin embedded, and sectioned. The presence of intracranial tumors, indicated by parenchymal and ventricular invasion, was assessed microscopically in multiple brain sections. The tumor proliferation activity was examined by Ki67 immunoreactivity using a Ki-67 monoclonal antibody (Lab Vision, Fremont, CA). OTX2 immunoreactivity was detected with biotinylated anti-human OTX2 antibody (BAF1979; R&D, Minneapolis, MN), followed by HRP-conjugated secondary antibody.

Immunoblotting

OTX2 was detected with biotinylated anti-human OTX2 antibody (BAF1979, R&D, Minneapolis, MN) and HRP-rabbit anti-goat IgG secondary antibody (Zymed, San Francisco, CA). MYC and GAPDH were detected with 9E10 (sc-40; Santa Cruz, CA) and FL-335 (sc-25778; Santa Cruz, CA), respectively, and enhanced chemiluminescence anti-rabbit IgG secondary antibody (Amersham Biosciences, Buckinghamshire, England).

siRNA and shRNA of OTX2 and MYC

Medulloblastoma cells were plated at 2×10^5 cells per well into a 6-well plate and transfected with siRNA or shRNA using Lipofectamine 2000 (Invitrogen). siRNA and shRNA sequences used are described in the Supplemental Methods section.

Animal survival studies

Approximately 10^7 D425MED cells were transfected with scrambled-shRNA or OTX2-shRNA, suspended in 10 µl of PBS, and stereotactically introduced into the right cerebral hemisphere of a BALB/c athymic nu/nu mouse. Mice were euthanized at the first sign of neurologic dysfunction. The date when each mouse became symptomatic was recorded to calculate the time-to-event. Standard Kaplan-Meier estimates were generated for each time-to-event, and groups were compared with log-rank tests.

Luciferase assays

For luciferase assays, we obtained the pBV-del-3-Luc plasmid, comprised of a 1.1-kb genomic fragment encompassing the *MYC* promoter inserted upstream of a minimal promoter and the firefly luciferase reporter gene in the pBV-Luc plasmid (Supplementary Fig 3*B*) (31). Medulloblastoma cells were seeded at 1×10^5 cells per well in 48-well plates for 24 h, and

then each well was cotransfected with 0.8 µg of firefly luciferase reporter plasmid pBV-del-3-Luc, the internal control plasmid pRL-CMV, and 0.8 µg of pEGFP-OTX2 or the control vector pEGFP-N1. Dual-Luciferase Reporter assays were performed according to the manufacturer's protocol (Promega, Madison, WI).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using a ChIP Assay Kit (Upstate Cell Signaling Solutions, Lake Placid, NY) according to the manufacturer's protocol. Immunoprecipitation was performed overnight at 4°C with biotinylated anti-human OTX2 antibody (R&D, Minneapolis, MN). Immunoaffinity-enriched and control DNA fragments were then purified for DNA PCR amplification.

Results

Recurrent *OTX2* focal gains and overexpression in medulloblastomas

To determine the frequency of *OTX2* genetic alterations in a large cohort of primary tumors, we utilized high-density SNP arrays to perform a genome-wide search for genetic changes in 11 medulloblastoma cell lines and 201 primary medulloblastomas (27). The most frequent target of focal gain among the 212 samples was a region mapping to chromosome 14q23, detected in ~10% (21/212) of the medulloblastomas (Fig 1*A*). This locus exhibited either focal (ie <1 Mb) or large (ie 14q gain) genomic gain in a total of ~21% (9.9% and 11.3%, respectively) of cases profiled. Examination of the minimal common region gained in these samples using the UCSC Genome Browser (NCBI Build 35, hg17; http://genome.ucsc.edu/) revealed that the single gene *OTX2* was completely located within this minimal region of chromosomal gain (Fig 1*A*). OTX2 gain of copy number indicated by SNP arrays was verified by Real-Time PCR on genomic DNA of selected medulloblastoma samples (Supplementary Fig 1). The highly specific gain of *OTX2* copy number in a large proportion of medulloblastomas implies that *OTX2* gain is a driver event that confers a selective advantage during clonal expansion of this neoplasm.

In addition to harboring focal gains and activating mutations, oncogenes are commonly overexpressed to activate tumorigenic pathways. To determine the prevalence of OTX2 mRNA overexpression, we utilized Affymetrix exon arrays to measure OTX2 mRNA level in 9 normal fetal cerebella, 5 normal adult cerebella, and 103 primary medulloblastomas. We found that fetal cerebellum expressed OTX2 at a modestly higher level than adult cerebellum; however, among the 103 medulloblastomas, 76 tumors (74%) expressed OTX2 at significantly (ie > two-fold) higher levels than fetal or adult cerebellum (P < 0.001, Student's T-Test) (Fig 1*B* and *C*).

To understand *OTX2* amplification at the cellular level, we performed dual-color FISH to validate *OTX2* amplification in three medulloblastoma cell lines previously identified to bear *OTX2* amplifications. We noted *OTX2* genomic amplification as double-minute chromosomes in D425MED, D458MED, and D487MED cell lines (Supplementary Fig 2, panels a–c). Moreover, increased *OTX2* copy number was also observed in the primary tumors from which the medulloblastoma cell lines D425MED and D487MED were derived (Supplementary Fig 2, panels d–e).

Medulloblastomas can be classified into distinct molecular subgroups based on gene expression signatures (28,32,33). Our clustering analysis of a large cohort (n=103) of tumors based upon Affymetrix exon arrays previously revealed four distinct medulloblastoma subtypes: tumors expressing a molecular signature of Wnt pathway activation, those exhibiting a signature of Shh pathway activation, and those bearing two additional, distinct molecular signatures

designated Group C and D tumors (28). Group C and D tumors as well as those with Wnt pathway activation are typically of the classic histopathological subtype, whereas tumors with a Shh molecular signature are often of the nodular/desmoplastic histopathological subtype (32,33). Examination of *OTX2* focal gains among medulloblastoma subtypes reveals that this event is limited to Group C and D tumors (Figure 2*A*). Additionally, OTX2 is differentially expressed in the molecular subgroups of medulloblastoma ($P < 0.001$, One-way ANOVA), with the most aberrant expression observed in Group C and D tumors as well as tumors with a Wnt pathway signature (Figure 2*B*).

OTX2 gain of copy number associates with anaplastic features and a worse patient outcome

To understand the clinical implication of OTX2 activation in medulloblastomas, we collected medulloblastoma samples with linked clinical data and studied *OTX2* genetic status in these cases using FISH. A total of 39 patients with complete clinical data were available for survival analysis on the basis of the FISH data, including 20 with tumors of classic or nodular histology and 19 with tumors of moderate to severe anaplasia. Classic and desmoplastic/nodular sections consistently demonstrated no evidence of increased *OTX2* copy number (0/20). However, a significant number of tumors with moderate and severe anaplastic characteristics displayed increased *OTX2* copy number (12/19, $P < 0.05$) (Fig 3*A*). Only a subpopulation of cells in those samples revealed increased copy number, with *OTX2* copy number ranging from 3 to 24 per individual cell (mean $= 2.3 \pm 1.7$). Among the 39 patients, 12 patients who had gain of *OTX2* copy number in their tumors had a shorter overall survival compared to those with diploid *OTX2* copy number ($P = 0.005$; Fig 3*B*). The 5-year survival estimate is 46% (95% CI: 24–87%) for the group with increased *OTX2* copy numbers and was nearly doubled (86%; 95% CI: 74–99%) for the group with normal *OTX2* copy numbers. Age stratification of this sample group revealed that survival of these patients mimics that of medulloblastoma patients in general, with age < 3 years indicating poor prognosis ($P = 0.001$) (Figure 3*C*).

OTX2 promotes cell proliferation and tumorigenicity and is essential for medulloblastoma cell survival

In order to examine the transforming potential of OTX2, we transfected the pEGFP-OTX2 plasmid into non-neoplastic RK3E cells, an adenovirus E1A-immortalized epithelial line that has been widely used for neoplastic transformation studies (30). This assay reveals oncogenic activity independent of cell origin effects, which is suitable considering the cell origin of medulloblastomas harboring *OTX2* focal gains (Group C and D tumors) is unknown. OTX2 protein expression was detected by Western blotting (Fig 4*A*). Overexpression of OTX2 promoted a significant increase in colony formation (Fig 4*A*, $P < 0.05$). To assess the ability of OTX2 to promote *in vivo* tumor growth, we injected RK3E cells expressing either pEGFP-OTX2 or pEGFP-N1 into the cerebral hemispheres of *nu/nu* mice. We found that while all OTX2-expressing clones formed tumors and displayed high mitotic indices scored by Ki67 staining (Fig 4*A*), GFP-expressing clones formed tumors much less frequently and scored relatively lower for Ki67 staining ($P < 0.001$, Supplementary Table 1). To verify that the transformative ability of OTX2 relies upon its transcriptional activity rather than alternative functions, we introduced a loss of function mutation into the OTX2 homeodomain (HD) to abolish DNA binding (34) (Supplementary Fig 3*A*). We then transfected MHH-1 medulloblastoma cells with either OTX2 or its HD NAAIRS mutant [pEGFP-OTX2 (K)] and measured MTT activity. As expected, wild type OTX2 increased proliferation of MHH-1 cells, and the effect was greatly reduced in the NAAIRS mutant (Fig 4*B*).

We have previously shown that OTX2 knockdown reduces viability of medulloblastoma cell lines (15). To determine if OTX2 expression is required for tumor maintenance *in vivo* as well as *in vitro*, we carried out RNA interference experiments in medulloblastoma xenografts. First, we utilized OTX2 siRNA or shRNA to confirm that OTX2 knockdown inhibited cell growth

or survival in an expanded cohort of *OTX2*-expressing medulloblastoma cell lines (Supplementary Fig 4). We then applied the pSUPER vector system to stably suppress endogenous OTX2 expression in D425MED cells (Fig 4*C*) and examined whether downregulation of OTX2 expression affected survival in nude mice bearing intracranial xenografts. Although tumors were detected from both the OTX2-knockdown group and the control group, reducing OTX2 expression significantly increased the survival time of the animals from 22.6 ± 4.7 days to 30.8 ± 5.9 days ($P = 0.0061$, Fig 4*C*).

MYC expression is regulated by OTX2

Amplification and overexpression of the oncogene *MYC* are also common in medulloblastoma, particularly in those exhibiting anaplastic histopathological features (35). Therefore we performed biochemical studies to investigate the relationship between OTX2 and MYC.

We first examined the correlation between OTX2 and MYC expression by Western blot in medulloblastoma cell lines that do not have *OTX2* or *MYC* amplification. We observed that detectable MYC expression level was limited to cell lines expressing OTX2 (Fig 5*A*). Using serial analysis of gene expression (SAGE) data from the Cancer Genome Anatomy Project, we found that presence of OTX2 mRNA correlated with the presence of MYC mRNA in medulloblastomas ($P = 0.0140$; Supplementary Fig 5).

To determine whether OTX2 is required for MYC expression in medulloblastoma, we applied OTX2-specific siRNA to knock down OTX2 expression in medulloblastoma cell lines and found that MYC expression was also downregulated by the OTX2 siRNA (Fig 5*B*). The complementary experiment in which MYC is knocked down by siRNA resulted in cell growth inhibition (Fig 5*C*) but not downregulation of OTX2 (Fig 5*D*). We then utilized a luciferase assay to demonstrate that OTX2 upregulates MYC via *cis*-acting elements in its promoter (Fig 6*A*), which contains multiple putative OTX2 binding sites (Supplementary Fig 3*B*). Finally, chromatin immunoprecipitation of fragments enriched with an anti-OTX2 antibody in cell lines expressing both MYC and OTX2 revealed direct binding of endogenous OTX2 to the *MYC* promoter in the context of native chromatin (Fig 6*B*).

Discussion

Medulloblastoma patients have widely disparate and often unpredictable clinical outcomes. It is therefore important to develop better predictive molecular diagnostic tools that could be used in clinical practice to better define prognosis and guide therapy. In the current report, the investigation of *OTX2* genetic status and its pathogenic roles in medulloblastoma oncogenesis have revealed that *OTX2* genetic alteration is associated with tumor progression and poor survival and thus may have diagnostic value. Furthermore, we have demonstrated a requirement for OTX2 in medulloblastoma viability, potentially revealing a novel therapeutic target in this tumor. We have also identified *MYC* as a downstream target gene of OTX2. This study advances our knowledge of the pathogenesis of medulloblastoma and has great potential to lead to significant contributions to the therapeutic intervention of this frequently lethal cancer.

The specific and recurrent focal gain of OTX2 represents a driver event in medulloblastoma tumorigenesis. *OTX2* copy number gain is limited to tumors not bearing a molecular signature of Shh or Wnt pathway activation and thus represents an oncogenic event in tumor subtypes whose molecular progressions are currently unclear. The Group C and D tumors that distinctly harbor *OTX2* copy number gains are typically of the classic histopathological subtype (22, 23). Considering that OTX2 gain is also associated with the presence of anaplastic features, it would be expected that there would be substantial overlap between Group C or D molecular signatures and the presence of anaplasia. It has been described previously that anaplastic

features can be present in variable degrees of severity and extent among the medulloblastoma histopathological subtypes (36).

Using FISH, we found that *OTX2* copy number varies widely among tumor cells, suggesting that *OTX2* gain of copy number is a late event that is selected for within the tumor as a step of tumor progression. This model is consistent with the association of *OTX2* copy number gain with worse patient survival as well as the presence of anaplastic features, which has been proposed to result from the progression of less aggressive tumors (37).

It remains a challenge to identify the patients with unfavorable prognoses who may benefit from the most aggressive treatment. Patient stratification is particularly important to medulloblastoma due to the deleterious effects of aggressive treatment in young patients. The Kaplan-Meier analysis revealed a significant association between increased *OTX2* copy number and worse survival. Similar to other study populations, this group of patients also demonstrated a clear association between poor survival and age <3 years, a well-known negative predictive factor for medulloblastoma. These findings support the possible use of *OTX2* genetic status as an important marker for prognosis. Also, OTX2 status may improve stratification schemes for current treatment protocols. de Haas et al. did not observe a similar correlation between OTX2 expression and outcome, which suggests that copy number gain is a more faithful indicator of poor prognosis than overexpression alone (38).

Although mutation and copy number analysis of tumor specimens has revolutionized our understanding of recurrent molecular events in cancer, it is important to confirm oncogenic activity by functional studies. Here we have demonstrated *in vitro* and *in vivo* that OTX2 promotes cell growth and enhances tumorigenicity, and that medulloblastoma cells rely on OTX2 transcriptional function for their aggressive tumorigenic phenotype. Therefore, we have demonstrated that the highly prevalent overexpression of OTX2 in medulloblastoma has functional significance.

Recent advances in cancer genetics and the development of mouse models have shown a relationship between developmentally associated molecular pathways and medulloblastoma pathogenesis (39). The current study supports previous work implicating *OTX2* as one such developmental gene involved in tumorigenesis (16,17). While medulloblastomas arising from oncogenic Shh signaling arise from committed granule cell precursors (40,41), the cell origins of Group C and D medulloblastomas (which distinctly harbor *OTX2* focal gains) are currently unknown. During normal cerebellum development, OTX2 is expressed in the prospective cerebellum as well as emergent and migrating granule cell precursors (20,38). Mouse models have demonstrated that OTX2 coordinates lineage differentiation in the central nervous system, promoting generation of dopaminergic neurons and, in some domains, repressing lineage commitment to granule cell and serotonergic neural lineages (42,43). Understanding the potential of OTX2 to facilitate tumorigenesis of particular cerebellum lineages will yield considerable insight into the cell origin of Group C and D medulloblastomas.

The molecular classification of medulloblastoma using gene expression signatures and select genomic features has been reported by three independent laboratories to date: Gilbertson (33), Kool (32), and ours (28). In all of these studies, very similar bioinformatics was employed. In each case, class discovery was achieved by performing unsupervised hierarchical clustering (Pearson correlation) using the most differentially expressed genes in the dataset. Thompson *et al* and Kool *et al* each identified 5 molecular subgroups (based upon 46 and 62 primary tumors, respectively), whereas we used 90 tumors and identified 4 molecular subgroups. The disparity between the 5 versus 4 subgroups pertains to what we have designated Group C and Group D, which are equivalent to what Kool *et al* referred to as C, D, and E. We have since analyzed a larger cohort of our own 103 cases, and again generated the same 4 molecular

subgroups with extremely high confidence (≥97% reproducibility; Northcott *et al*, in preparation). The larger sample cohort and more robust array platform (ie Affymetrix exon arrays vs U133 arrays) utilized in the current study provides increased power and statistical confidence in class discovery. Further application of the proposed molecular clustering strategies will reveal the most faithful method of tumor classification.

This study provides important insights into the mechanisms by which medulloblastomas develop through the dysregulation of *OTX2*, a gene normally silenced in adult cerebellum. Furthermore, these results establish OTX2 as a potential therapeutic target, the inhibition of which could result in tumor cell growth inhibition. For the future development of targeted therapies, it will be important to perform detailed molecular studies of the oncogenic pathways driven by OTX2 as well as the pathways maintaining its expression in tumors. These studies will aide the development of molecular diagnostic and therapeutic strategies for patients whose tumors present with this molecular hallmark. Once such strategies become available, there is promise that in tumors with OTX2 expression, the dysregulated signaling pathway can be corrected by specifically reducing OTX2 expression or inhibiting other components of this pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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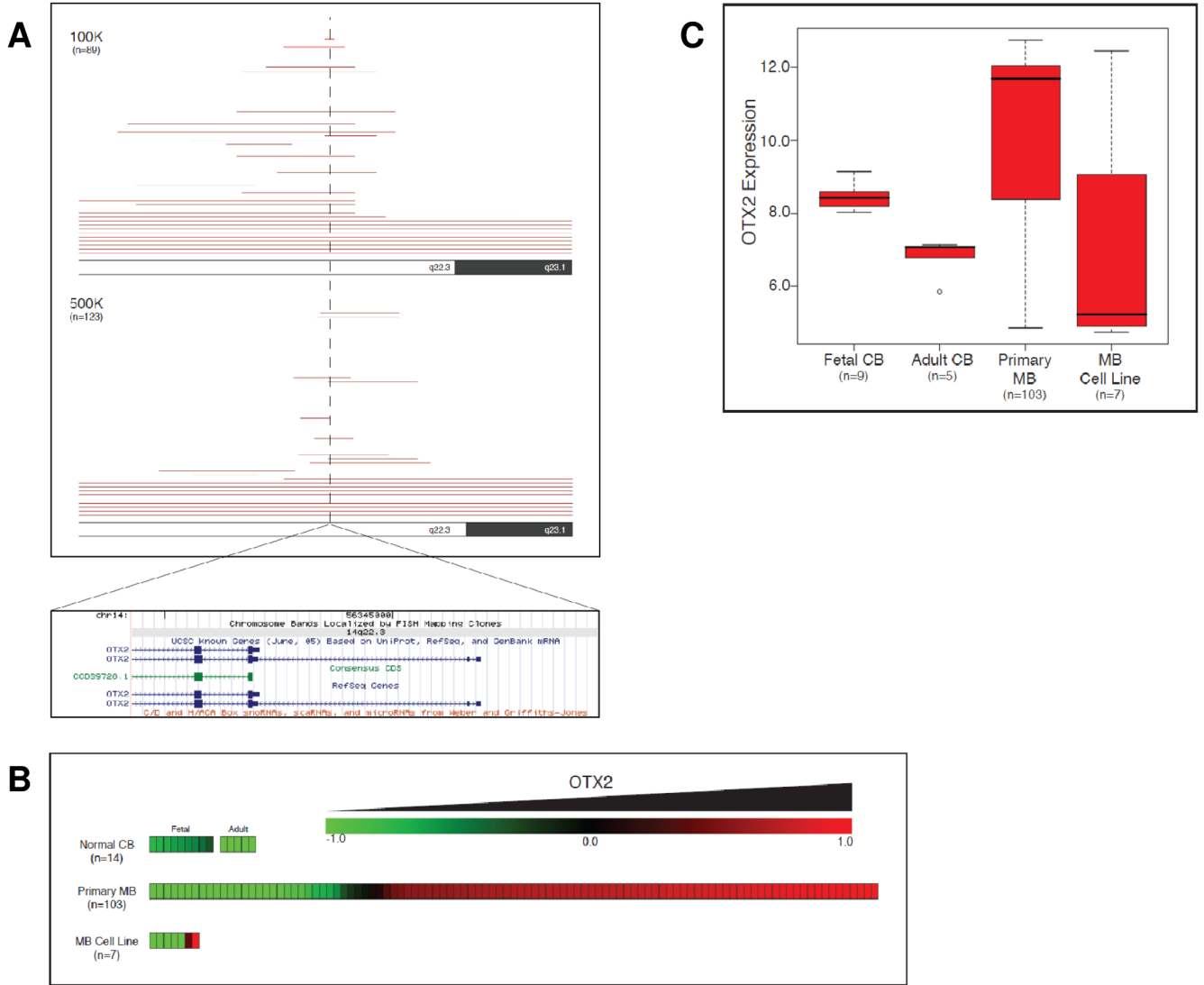
Figure 1. Frequent gain and overexpression of *OTX2* in medulloblastomas
(*A*) OTX2 copy number status in 212 medulloblastomas (201 primary MBs and 11 MB cell lines) was inferred using Affymetrix SNP arrays (100K and 500K). Each red line shown in the ideogram represents copy number gain for a single sample in the region shown. (*B*) Affymetrix exon arrays were used for gene expression profiling of 9 fetal cerebella, 5 adult cerebella, 103 primary medulloblastomas, and 7 medulloblastoma cell lines. OTX2 expression levels are shown as a heatmap. (*C*) Box and whisker plot representing distribution of OTX2 expression level within normal and MB sample groups.
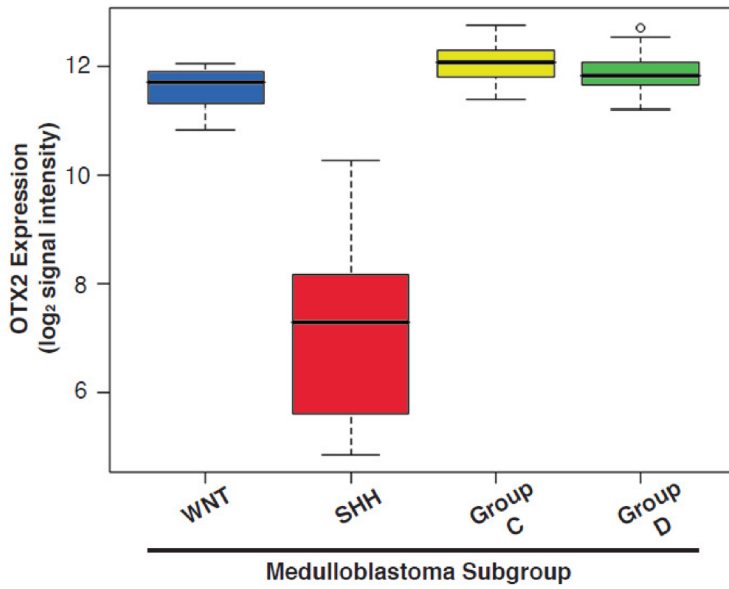
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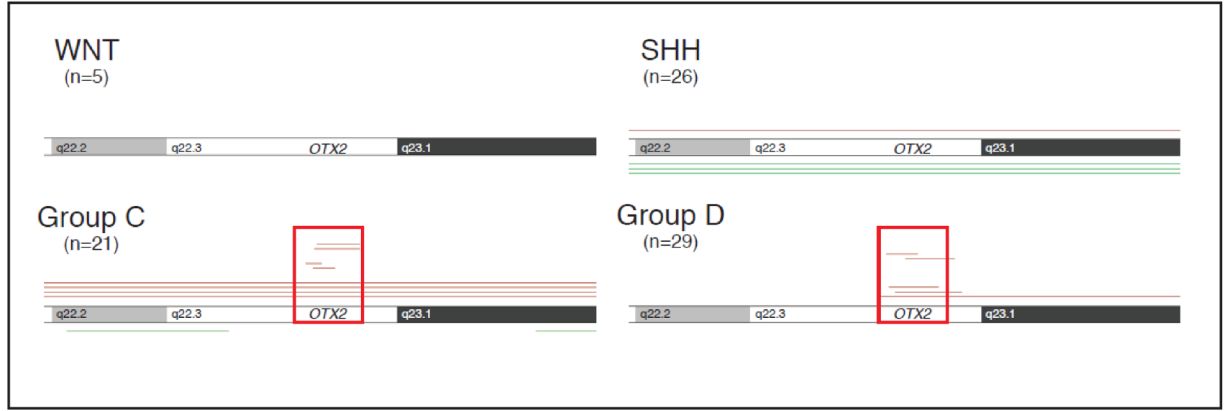
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Figure 2. *OTX2* **gain and overexpression are specific to medulloblastoma subgroups**
A subset of the primary medulloblastomas (n=81) analyzed in Figure 1 were classified into four molecular subgroups based upon gene expression signatures obtained from exon arrays. (*A*) Focal gain of *OTX2* (designated in red) is limited to Group C and D medulloblastomas. The minimal region of gain (~10.5 kb) is boxed. (*B*) OTX2 mRNA overexpression is highest in Group C and D tumors, as well as those expressing a signature of Wnt pathway activation.
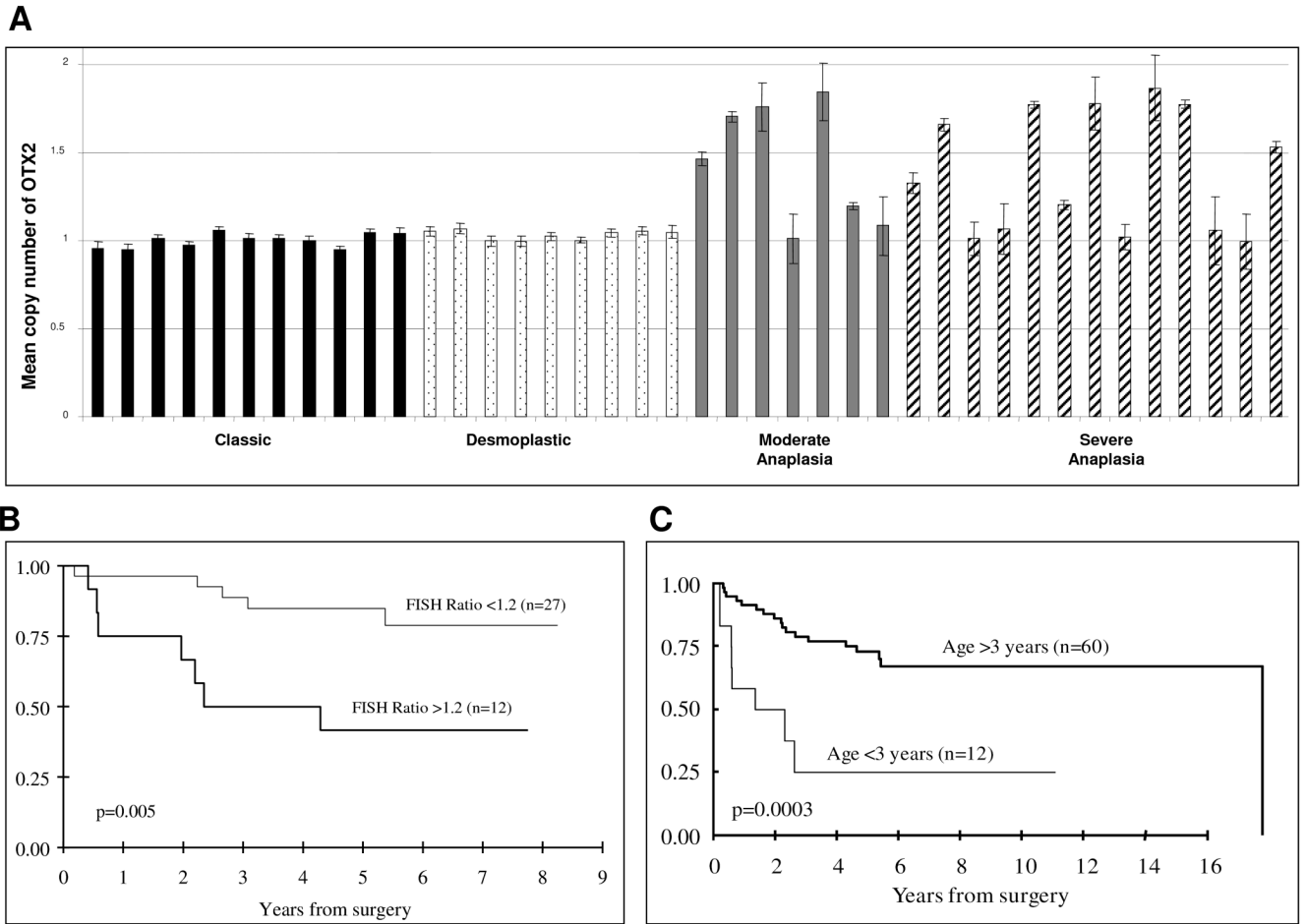
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Figure 3. Interphase FISH of *OTX2* in primary medulloblastoma showing that *OTX2* copy number gain is associated with tumor anaplasia and poor patient survival
(*A*) Increased *OTX2* copy number is seen in primary medulloblastoma tumors with anaplastic features. The Y axis is the ratio of the mean copy number of *OTX2* to the control chromosome 14 probe in each cell of each sample. (*B*) Kaplan-Meier survival analysis of patients stratified by mean OTX2 copy number. (*C*) Kaplan-Meier survival analysis of patients stratified by age.
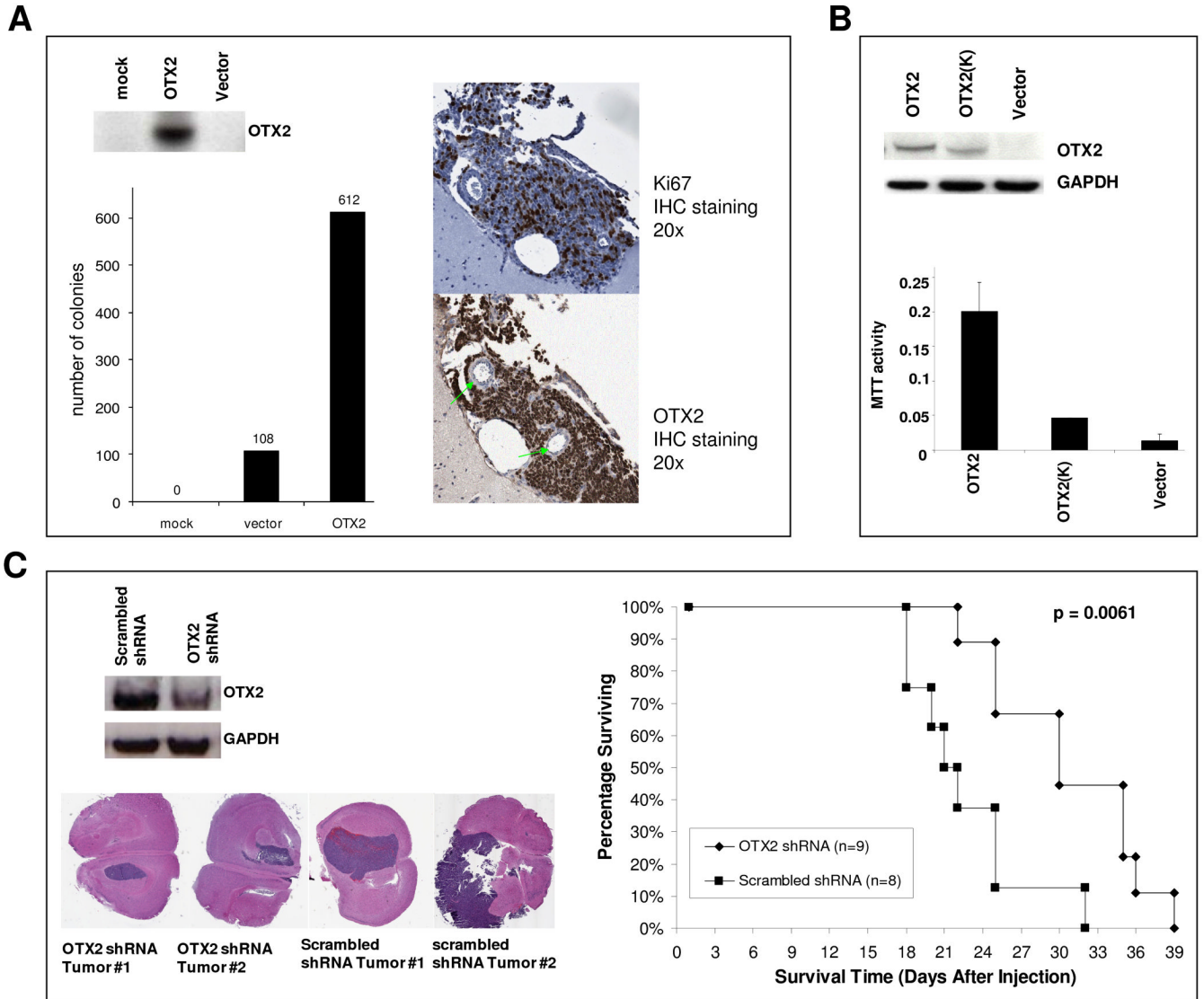
Figure 4. OTX2 promotes cell proliferation *in vitro* and is required for tumor maintenance *in vivo*
(*A*) RK3E cells were transfected with pEGFP-N1 or pEGFP-OTX2 and colony formation potential was determined. Tumors derived from intracranial xenografts of stably transfected RK3E cells were stained for Ki67 and OTX2, and representative samples are pictured. Green arrows identify intra-tumor blood vessels. (*B*) MHH-1 cells were transfected with pEGFP-N1, pEGFP-OTX2, or pEGFP-OTX2 (K). After three doubling times, proliferation was determined with an MTT assay. (*C*) D425MED cells derived from OTX2 shRNA knockdown or scrambled shRNA were injected into the right cerebral hemisphere of mice. Representative H & E-stained tumor sections from each group are pictured.
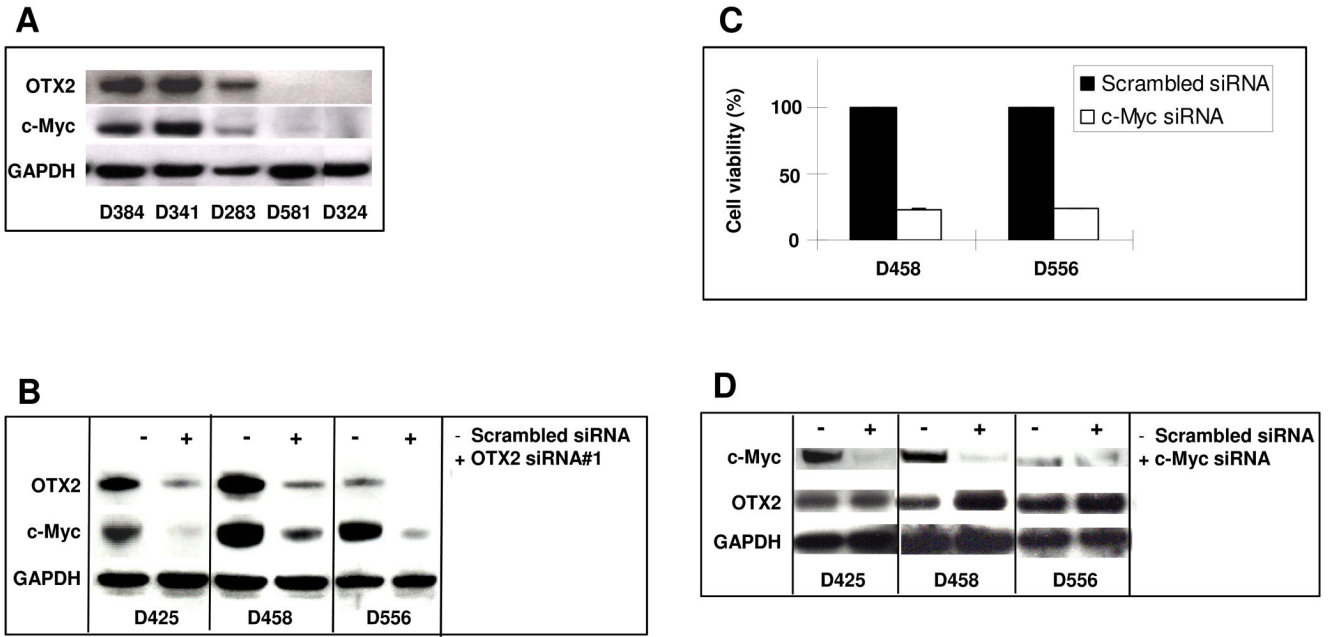
Figure 5. *MYC* is a target gene of OTX2 in medulloblastoma cell lines
(*A*) Expression of endogenous OTX2 and MYC in medulloblastoma cell lines demonstrated by Western blotting. (*B*) Medulloblastoma cell lines were treated with OTX2 siRNA (+) or scrambled siRNA (−), and protein expression of OTX2, MYC, and GAPDH was detected by Western blotting. (*C*) Cells were treated with MYC siRNA or scrambled siRNA and after three doubling times assessed for viability with the MTT assay. (*D*) Medulloblastoma cell lines were treated with MYC siRNA (+) or scrambled siRNA (−), and protein expression of OTX2, MYC, and GAPDH was detected by Western blotting.
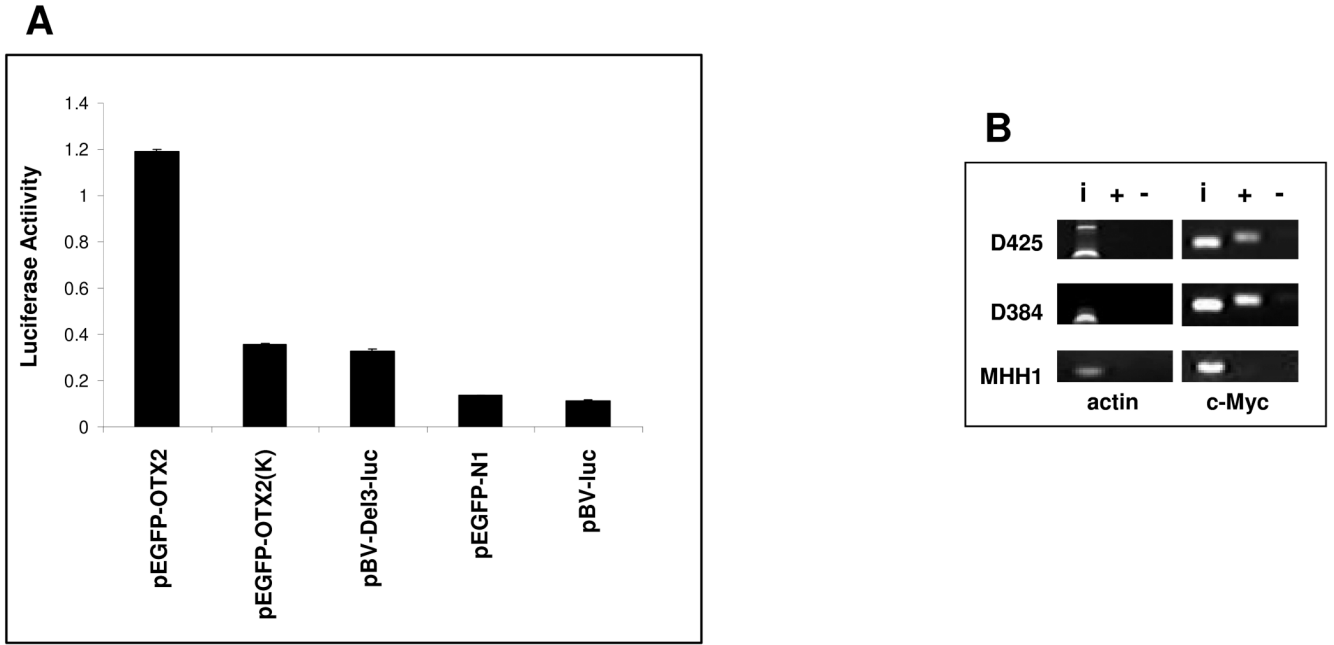
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Figure 6. The OTX2 homeodomain is required for OTX2-mediated MYC transcription
(*A*) MHH-1 cells were cotransfected with the *MYC* promoter reporter construct (pBV-Del-3-
Luc) and either pEGFP-N1, pEGFP-OTX2 or pEGFP-OTX2 (K), and then the relative
luciferase reporter activity was measured. (*B*) OTX2 ChIP and PCR analyses were used to
validate binding of endogenous OTX2 to the promoter region of *MYC* (primers were designed
to span the region 375 bp directly upstream of the *MYC* TSS) in OTX2-expressing cell lines.
D425MED and D384MED are OTX2-expressing medulloblastoma cell lines, whereas MHH1
does not express OTX2. i, input of total DNA from cell lysates; +, OTX2 antibody
immunoaffinity-enriched DNA; −, IgG control antibody immunoaffinity-enriched DNA.