

The Role of Otx2 in Bypassing Restrictions of Hindbrain Progenitor Cell Proliferation  
and the Mechanisms of its Dysregulation in Medulloblastoma

by

Matthew Jeremy Wortham

Department of Pathology  
Duke University

Date: \_\_\_\_\_

Approved:

\_\_\_\_\_  
Hai Yan, Supervisor

\_\_\_\_\_  
Robin Bachelder

\_\_\_\_\_  
Oren Becher

\_\_\_\_\_  
Sridharan Gururangan

\_\_\_\_\_  
Salvatore Pizzo

Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor  
of Philosophy in the Department of  
Pathology in the Graduate School  
of Duke University

2012

ABSTRACT

The Role of Otx2 in Bypassing Restrictions of Hindbrain Progenitor Cell Proliferation  
and the Mechanisms of its Dysregulation in Medulloblastoma

by

Matthew Jeremy Wortham

Department of Pathology  
Duke University

Date: \_\_\_\_\_

Approved:

\_\_\_\_\_  
Hai Yan, Supervisor

\_\_\_\_\_  
Robin Bachelder

\_\_\_\_\_  
Oren Becher

\_\_\_\_\_  
Sridharan Gururangan

\_\_\_\_\_  
Salvatore Pizzo

An abstract of a dissertation submitted in partial  
fulfillment of the requirements for the degree  
of Doctor of Philosophy in the Department of  
Pathology in the Graduate School of  
Duke University

2012

Copyright by  
Matthew Jeremy Wortham  
2012

## Abstract

Medulloblastoma is the most common malignant brain tumor in children. The understanding of the genetic alterations in this tumor is emergent, and many such genetic driver events have yet to be functionally-characterized. Our studies have sought to understand the causes and consequences of OTX2 dysregulation in established medulloblastomas and in its putative cellular origins. Using a tumor genetic approach, we have uncovered frequent *OTX2* copy number gains driving expression of this oncogene in a subset of medulloblastomas. However, OTX2 is frequently expressed in medulloblastomas independent of genomic copy number gain, and we thus sought to understand the transcriptional regulation of this gene in these tumors. We have found that chromatin accessibility, promoter DNA methylation, and activity of a distal downstream enhancer is distinct between OTX2-expressing and -nonexpressing medulloblastomas. Notably, autoregulation serves to maintain OTX2 expression in some medulloblastomas, whereas DNA methylation actively suppresses OTX2 in tumors not expressing this gene. Finally, we describe the effect of expressing *Otx2* (the mouse homolog of OTX2) aberrantly in the developing mouse hindbrain, revealing that *Otx2* disrupts spatiotemporal restrictions of neuronal progenitor cell proliferation. The effect of *Otx2 in vivo* is transient, with ectopically-proliferating cells give way to differentiated neurons. We found that OTX2 expression was not able to give rise to high penetrance

medulloblastoma when combined with *P53* deletion or double heterozygosity for *P53* and *PTEN*. Thus, although *Otx2* alters migration and proliferation dynamics of hindbrain neuronal progenitor cells, further studies are needed to identify the genetic alterations that cooperate with this oncogene to give rise to medulloblastoma.

# Contents

Abstract .....	iv
List of Tables .....	xi
List of Figures .....	xii
Acknowledgements .....	xv
1. Introduction .....	1
1.1 Permissions and collaborations .....	1
1.2 The emerging genetic landscape of medulloblastoma reveals OTX2 as a candidate for non-Shh medulloblastoma animal modeling and therapeutic targeting .....	1
2. Characterizing <i>OTX2</i> genetic alterations and functional consequences in established medulloblastomas .....	6
2.1 Introduction: Recurrent genetic alterations in Group 3 and 4 medulloblastoma subtypes .....	6
2.2 Methods .....	8
2.2.1 Medulloblastoma samples .....	8
2.2.2 Cell culture, transfections, and xenografts .....	8
2.2.3 Retrovirus production and infection .....	10
2.2.4 Western blotting and immunohistochemistry .....	10
2.2.5 ChIP-chip and expression analysis of OTX2 siRNA-treated cells .....	10
2.3 Prevalence and subtype specificity of OTX2 copy number gain in medulloblastoma .....	11
2.4 Requirement for OTX2 in tumor maintenance .....	15
2.4.1 OTX2 is required for medulloblastoma proliferation <i>in vitro</i> .....	15

2.4.2 OTX2 is required for xenograft tumorigenicity .....	16
2.4.3 OTX2 silencing is a critical event in retinoic acid-induced growth suppression .....	18
2.5 Identification and classification of OTX2 transcriptional targets .....	20
2.6 Evidence of a role for OTX2 in tumorigenesis.....	24
3. Identifying the mechanisms of OTX2 overexpression in medulloblastoma .....	27
3.1 Introduction: the transcriptional dysregulation of oncogenes and tumor suppressors as key events in tumorigenesis.....	27
3.2 Methods .....	29
3.2.1 Cell culture, transfections, and gene expression studies .....	29
3.2.2 DNase hypersensitive (DHS) site mapping.....	31
3.2.3 Motif discovery.....	32
3.3 Mapping the chromatin accessibility landscape of the OTX2 locus.....	32
3.3.1 Establishing a genomewide chromatin accessibility profile in two OTX2-expressing cell lines.....	32
3.3.2 Assessing DHS site utilization across medulloblastoma cell lines using a qPCR-based assay of chromatin accessibility.....	35
3.4 Assessment of DHS sites for transcriptional regulatory activity.....	36
3.5 Identification of an insulator element repressing DHS 1.....	40
3.6 Characterization of trans-acting factors mediating enhancer activity of DHS 4... 41	
3.6.1 Identification of a minimally-required region of the DHS 4 enhancer .....	41
3.6.2 Scanning for protein binding motifs in the minimally-required region of DHS 4.....	42

3.6.3 Validation of an OTX2 autoregulatory loop in a distal transcriptional enhancer .....	45
3.7 Characterization of DNA methylation patterns associated with OTX2 silencing.	47
3.8 Characterization of the epigenetic modifications of relevant OTX2 regulatory elements during embryonic brain development.....	50
3.9 Identifying the mediator of retinoic acid-induced repression of OTX2 .....	53
3.10 A model for basal OTX2 overexpression in medulloblastoma .....	56
3.11 A model for retinoid-induced OTX2 repression in medulloblastoma.....	57
4. Characterizing the functional consequences of Otx2 overexpression in the postnatal hindbrain.....	59
4.1 Introduction: Animal modeling of medulloblastoma subtypes reveals cell type-specific effects of genetic alterations found in distinct tumor subgroups.....	59
4.2 Methods .....	62
4.2.1 Generation of a cre-inducible system for ectopic Otx2 expression <i>in vivo</i> .....	62
4.2.2 Animal breeding and maintenance.....	64
4.2.3 Tissue processing and histology .....	64
4.3 Validation of ROSA26 <sup>Lsl-Otx2</sup> mice .....	66
4.4 Characterizing the effects of Otx2 upon animal survival and hindbrain development.....	68
4.4.1 Effect of ectopic Otx2 expression upon survival.....	68
4.4.2 Effect of ectopic Otx2 expression upon hindbrain development .....	71
4.5 Investigation of the distinct effects of Otx2 upon neuronal progenitor cells.....	75
4.5.1 Identification of neuronal progenitor cells as being distinctly responsive to Otx2 overexpression.....	75

4.5.2 Validation of a migratory defect in cerebellar ectopia .....	78
4.5.3 Partial phenocopy of the focal hyperplasia phenotype in Math1:Hi-Otx2 mice .....	80
4.6 Determining the ability of co-occurring genetic alterations to cooperate with Otx2 to form medulloblastoma .....	82
4.6.1 Homozygous <i>P53</i> deletion does not induce highly-penetrant medulloblastoma in GFAP:Hi-Otx2 mice .....	82
4.6.2 Genetic, but not functional, evidence for cooperation of <i>PTEN</i> and <i>P53</i> heterozygous loss with <i>OTX2</i> alterations .....	85
4.6.3 The effect of Otx2 upon tumor promotion in medulloblastomas initiated by constitutive Shh pathway activation .....	88
5. Conclusions and future directions.....	94
5.1 OTX2 genetic alterations, overexpression, and its role in tumor maintenance .....	94
5.1.1 Spectrum of <i>OTX2</i> genetic alterations and overexpression across medulloblastoma subtypes .....	94
5.1.2 Critical transcriptional targets of OTX2 in medulloblastoma .....	95
5.2 The mechanisms of OTX2 dysregulation in medulloblastoma.....	97
5.2.1 Model for OTX2 dysregulation during medulloblastoma tumorigenesis.....	98
5.2.2 The role of CRX in retinoid-mediated repression of OTX2 .....	101
5.2.3 Cell populations in the developing brain utilizing the medulloblastoma- specific <i>OTX2</i> enhancer .....	102
5.3 The effect of aberrant Otx2 expression upon development of the hindbrain and its relationship to medulloblastoma tumorigenesis .....	104
5.3.1 Potential mechanisms of Otx2-induced migration and ectopic proliferation of hindbrain neuronal progenitor cells .....	104

5.3.2 Alternative and additional strategies of deriving Otx2-initiated medulloblastomas .....	106
5.4 Implications of OTX2 autoregulation for the phenotype observed in GFAP:Hi-Otx2 mice .....	109
5.5 Summary and closing remarks.....	110
Appendix.....	111
References .....	112
Biography .....	126

## List of Tables

Table 1: Functional categories of genes transcriptionally upregulated by OTX2 in medulloblastoma.....	23
Table 2: Functional categories of genes transcriptionally repressed by OTX2 in medulloblastoma.....	24
Table 3: Tumor formation rate and Ki67 indices of tumors and foci derived from RK3E intracranial xenografts.....	26
Table 4: Spectrum of tumors and survival for mice harboring various combinations of Otx2 expression and tumor suppressor deletions.....	84

## List of Figures

Figure 1: Copy number gains of chromosome 14 targeting <i>OTX2</i> in medulloblastoma..	13
Figure 2: Frequent <i>OTX2</i> expression in medulloblastoma.....	14
Figure 3: Subgroup-specific <i>OTX2</i> copy number gains and mRNA expression.....	15
Figure 4: <i>OTX2</i> is required for medulloblastoma cell proliferation.....	17
Figure 5: <i>OTX2</i> is required for medulloblastoma xenograft tumorigenicity.....	18
Figure 6: <i>OTX2</i> repression is required for the effect of retinoids upon medulloblastoma proliferation .....	19
Figure 7: <i>OTX2</i> regulates hundreds of genes in medulloblastoma.....	21
Figure 8 (continued): <i>OTX2</i> enhances tumorigenicity of immortalized RK3E cells.....	26
Figure 9 (continued): Chromatin accessibility landscape of medulloblastoma. ....	34
Figure 10 (continued): Chromatin accessibility and other structural characteristics of the <i>OTX2</i> locus in various <i>OTX2</i> -expressing cell types. ....	35
Figure 11 (continued): Chromatin accessibility of medulloblastoma DHS sites across various <i>OTX2</i> -expressing and -nonexpressing cell lines.....	38
Figure 12: Transcriptional activity of medulloblastoma DHS sites. ....	39
Figure 13: Insulator activity of DHS 3.....	41
Figure 14: Identifying the minimally-required fragment for DHS 4 activity .....	42
Figure 15: Identifying <i>trans</i> -acting regulators binding to Fragment A, the minimally-required sequence of DHS 4 .....	43
Figure 16: Identifying a DNase footprint at the <i>OTX2</i> binding motif in DHS 4 .....	44
Figure 17: Assessing the requirement of <i>OTX2</i> for DHS 4 enhancer activity.....	45
Figure 18: Assessing the ability of <i>OTX2</i> to enhance its own expression .....	46

Figure 19: Assessing binding of endogenous OTX2 to DHS 4 .....	47
Figure 20 (continued): Assessing the methylation status of the <i>OTX2</i> promoter in medulloblastoma.....	49
Figure 21: Promoter methylation actively represses OTX2 expression.....	49
Figure 22: Chromatin structure of the <i>OTX2</i> locus in the developing brain and adult cerebellum.....	51
Figure 23: Location and characteristics of medulloblastoma DHS sites .....	52
Figure 24 (continued): Dynamics of CRX expression and its putative binding site during retinoid treatment of medulloblastoma.....	55
Figure 25: CRX induction is required for retinoid-mediated OTX2 repression.....	56
Figure 26: Targeting strategy and composition of the inducible Otx2 expression construct.....	64
Figure 27: Validation of target construct integration and cre inducibility in ES cells.....	66
Figure 28: Validation of Otx2 expression in cerebella of GFAP:Hi-Otx2 mice.....	68
Figure 29: Validation of Otx2 expression in diverse cell types of the cerebellum .....	69
Figure 30: Survival of GFAP:Hi-Otx2 mice .....	70
Figure 31: Brain morphology of GFAP:Hi-Otx2 mice.....	71
Figure 32: Cerebellar morphology of GFAP:Hi-Otx2 mice.....	72
Figure 33: Focal hyperplasia in GFAP:Hi-Otx2 mice.....	73
Figure 34: Prevalence of ectopia in GFAP:Hi-Otx2 mice over time.....	74
Figure 35: Fate of ectopia in GFAP:Hi-Otx2 mice .....	75
Figure 36: Ectopia in GFAP:Hi-Otx2 mice resemble neuronal progenitor cells.....	77
Figure 37: Staining for cell lineage markers and Otx2 protein in ectopia.....	78

Figure 38: Spatial origin of ectopia .....	80
Figure 39: Restricting <i>Otx2</i> expression to <i>Math1</i> -expressing neuronal progenitor cells partially phenocopies <i>hGFAP-cre</i> induction of <i>Otx2</i> .....	81
Figure 40 (continued): Overall survival of mice expressing <i>Otx2</i> and lacking various tumor suppressors .....	85
Figure 41: Pairwise survival comparisons between mice expressing <i>Otx2</i> and lacking various tumor suppressors.....	85
Figure 42: Genetic alterations present in medulloblastoma subtypes and individual tumors harboring <i>OTX2</i> copy number gain.....	87
Figure 43: Preneoplastic lesions of ND2: <i>SmoA1</i> mice express <i>Math1</i> .....	89
Figure 44: Tumor latency in ND2: <i>SmoA1</i> <sup>+/-</sup> mice expressing low and high levels of <i>Otx2</i> .....	91
Figure 45: Pairwise survival comparisons between ND2: <i>SmoA1</i> <sup>+/-</sup> animals expressing various levels of <i>Otx2</i> .....	92
Figure 46: Histology of medulloblastomas from <i>SmoA1</i> <sup>+/-</sup> , <i>Math1:Hi-Otx2</i> and control mice. ....	93
Figure 47 (continued): Expression of developmental <i>OTX2</i> regulators in medulloblastoma.....	101

## Acknowledgements

I thank Hai Yan for guidance, mentorship, and support of the described studies and of continuing endeavors. I thank Robin Bachelder, Oren Becher, Sridharan Gururangan, and Sal Pizzo of my thesis committee for continued guidance and support.

I would like to thank our various collaborators for their generous and critical contributions. Medulloblastoma samples were processed for DNA copy number assessment and gene expression profiling by Michael Taylor and Paul Northcott at the Hospital for Sick Children at the University of Toronto. Their dedicated work and generous collaborations have served to advance the field towards a greatly improved understanding of medulloblastoma at the molecular level. I thank Greg Crawford and Lingyun Song for their contributions to the mapping of transcriptional regulatory elements, and specifically for DNase-seq preparation and analysis of medulloblastoma cell lines, and I thank Bum-Kyu Lee and Vishwanath Iyer for performing CTCF and Pol2 ChIP-seq analysis on medulloblastoma cell lines. I thank Rob Wechsler-Reya for extensive and continued training and mentorship, which critically contributed to the quality of the study of the *in vivo* effects of Otx2 and greatly influenced my subsequent research interests. I thank Cheryl Bock of the Duke transgenic mouse facility for support in generating the *ROSA26<sup>Lsl-Otx2</sup>* mice.

I thank Cory Adamson, Chunhui Di, Qun Shi, Chris Duncan, and Jianjun Li for their early studies of OTX2 function and direct involvement with data generation for Chapter 2, without which the subsequent studies would not have been possible. I thank Lailai Sun and Genglin Jin for critical contributions to the OTX2 mouse study. I thank past and present members of the Yan lab for helpful discussions and training, Yiping He for helpful and enjoyable discussions of the current work and ongoing studies, and Darell Bigner for his seminal contributions to brain tumor research and his leadership in supporting a strong research program through the Preston Robert Tisch Brain Tumor Center. I thank Roger McLendon, Lisa Ehinger, Diane Satterfield, Ahmad Rasheed, David Lister, and Charles Pegram at the Preston Robert Tisch Brain Tumor Center for helpful discussions and generous sharing of equipment and reagents. I thank Cathy Payne and Joey Webb for assistance with animal genotyping and Gerard Smith for excellent animal care. This work was supported by NIH grant R01CA118822, ACS Grant RSG-10-126-01-CCE, and a Voices Against Brain Cancer grant.

# **1. Introduction**

## ***1.1 Permissions and collaborations***

This dissertation contains reproductions of copyrighted material published elsewhere, with permission from each copyright holder if necessary, as described in the Appendix. This dissertation encompasses experiments and analysis performed by the candidate with technical and material contributions from colleagues and collaborators as described in the Acknowledgements.

## ***1.2 The emerging genetic landscape of medulloblastoma reveals OTX2 as a candidate for non-Shh medulloblastoma animal modeling and therapeutic targeting***

Medulloblastoma is the most common malignant brain tumor in children, manifesting in the cerebellum and occurring at a peak age of seven (Kleihues et al. 2002; Pietsch et al. 2004). These tumors are aggressive and tend to metastasize throughout the craniospinal axis, leading to significant morbidity due to excessive intracranial pressure and disruption of central nervous system (CNS) functions. Early symptoms of medulloblastoma include blurred vision, headaches, and vomiting, and untreated disease is nearly invariably lethal due to eventual failure of autonomic cycles. Accordingly, aggressive treatment regimens including resection, radiation, and traditional chemotherapy have been applied to combat the primary tumor and its metastases, achieving 5-year survival rates of 50-60% (Packer et al. 1991; Gajjar et al. 2004; Packer and Reddy 2004; Ray et al. 2004). However, in addition to the common

morbidities associated with aggressive cancer therapy, long-term side effects are profound in these young patients who additionally suffer from endocrine disorders, growth impairment, mutism, reduced intellectual development, and a risk for secondary tumors (Hoppe-Hirsch et al. 1990; Ris et al. 2001; Stavrou et al. 2001; Xu et al. 2004; Packer et al. 2012). Despite extensive cytotoxic treatment, medulloblastoma remains one of the main causes of cancer deaths in young children (Zakhary 2001). Thus, therapeutic strategies exhibiting increased efficacy and reduced comorbidities would provide tremendous benefit for medulloblastoma patients.

Targeted therapeutics have shown promise to achieve this end in the treatment of a variety of tumors. Limiting drug targets to those unique to tumors could substantially reduce side effects relative to radiation and nonselective chemotherapy. For example, promyelocytic leukemias depend upon the PML-RAR (Promyelocytic Leukemia-Retinoic Acid Receptor) fusion protein for tumor maintenance, and activation of the chimeric receptor with 13-*cis* retinoic acid results in a nearly complete cure rate with minimal side effects (Fenaux et al. 1995). However, the development of targeted therapeutics to most other tumors has been slow to emerge largely due to a poor understanding of the genomic events distinguishing tumors (and their various subtypes) from normal cells. As proof of principle, medulloblastomas exhibiting hyperactivation of the Shh pathway have been shown to respond to Shh pathway inhibitors (Rudin et al. 2009). However, activation of this pathway occurs in only a subset of medulloblastomas

(Northcott et al. 2011), and thus an improved understanding of the pathways driving non-Shh medulloblastomas is critical to the design of targeted therapeutics for these tumors.

Due to advanced technology and progressively-expanding tissue banks, substantial progress has been achieved in the characterization of the medulloblastoma genome (Northcott et al. 2011; Jones et al. 2012; Northcott et al. 2012; Pugh et al. 2012; Robinson et al. 2012). These studies have highlighted a number of complementary observations about the medulloblastoma genome. First, medulloblastoma comprises at least four molecular subtypes based on gene expression profiling, and each subtype exhibits distinct survival tendencies, patient demographics, and molecular alterations (described in Chapter 2.1). One of the most frequent gene-targeted alterations identified in the medulloblastoma genome is gain of copy number of *orthodenticle homolog 2* (*OTX2*), observed in 21% of primary tumors, in particular those not expressing a signature of Sonic Hedgehog (Shh) pathway activation (Adamson et al. 2010). As such, our lab has pursued the characterization of *OTX2* gene function to understand its role in medulloblastoma pathogenesis, revealing that it promotes tumorigenicity and is required for tumor maintenance (Adamson et al. 2010).

As a potential target for selective therapeutics, we have sought to define the mechanisms whereby *OTX2* is aberrantly transcribed in these tumors, with the goal of identifying strategies of suppressing *OTX2* expression and thus its function. This

approach is exemplified by the observation that retinoids can suppress OTX2 during both embryonic patterning (Simeone et al. 1995) and in medulloblastoma (Di et al. 2005; Bai et al. 2010). The failure of retinoids to robustly suppress xenograft growth (Spiller et al. 2008; Bai et al. 2010) suggests that identifying alternative strategies of repressing OTX2, or understanding the intermediaries of retinoid-induced OTX2 repression, would be beneficial to the design of OTX2-based therapeutics for medulloblastoma.

Additionally, we are interested in understanding how OTX2 contributes to the formation of non-Shh medulloblastomas, where it is uniquely expressed. Although a role for OTX2 in the maintenance of established tumors is clear, the potential for OTX2 to contribute to tumor initiation or tumor promotion is unknown. Broad expression of OTX2 within medulloblastoma samples is suggestive of a potential for the tumor bulk to have inherited OTX2 expression from the tumor cell of origin. Reminiscent of other developmental pathways involved in medulloblastoma initiation (Thomas and Capecchi 1990; Wechsler-Reya and Scott 1999; Yang et al. 2008; Gibson et al. 2010), OTX2 plays important roles in development of the hindbrain (Fossat et al. 2006), particularly in patterning the midbrain-hindbrain junction (Acampora et al. 1997; Broccoli et al. 1999) and in mediating foliation of the cerebellum (Fossat et al. 2006). OTX2 function exhibits strong contextual dependence, likely mediating particular cellular phenotypes through the integration of various signals of regionalization and developmental stage. Following neural tube regionalization, OTX2 functions cell-autonomously to coordinate the

proliferation and differentiation of neuronal progenitor cells contributing to mesencephalon-derived tissues such as the inferior and superior colliculi and the posterior cerebellum (Hidalgo-Sanchez et al. 1999; Vernay et al. 2005; Fossat et al. 2006; Omodei et al. 2008). Specifically, OTX2 can suppress the generation of granule and serotonergic neurons and can promote the generation of dopaminergic neurons (Omodei et al. 2008) in a location-dependent manner. While OTX2 promotes proliferation of ventral midbrain neuronal progenitor cells (Vernay et al. 2005), it suppresses proliferation in the thalamus (Puelles et al. 2006). Emerging granule neuron precursors (GNPs) of the posterior external granule layer (EGL) express OTX2 (Simeone et al. 1993; Frantz et al. 1994), and maintain OTX2 expression into adulthood (Fossat et al. 2006). Notably, OTX2 does not uniformly contribute to cell proliferation or other phenotypes critical to tumorigenesis or tumor promotion such as is observed for various classical oncogenes. These context-specific functional roles of OTX2 underscore the importance of studying its oncogenic activity in the anatomical origin of medulloblastoma, the developing hindbrain. As such, for studies of the role of OTX2 in transformation, we sought to determine the functional consequences of aberrant OTX2 expression in this particular developmental context.

## **2. Characterizing *OTX2* genetic alterations and functional consequences in established medulloblastomas**

### ***2.1 Introduction: Recurrent genetic alterations in Group 3 and 4 medulloblastoma subtypes***

Over the course of the last decade, the understanding of the genetic alterations that contribute to medulloblastoma have advanced considerably. While histological variants of medulloblastoma (e.g. nodular/desmoplastic, classic, and large cell/anaplastic) (Kleihues et al. 2002) and variability of survival outcomes (Eberhart and Burger 2003) have been appreciated for some time, the extent of heterogeneity among medulloblastomas has only recently been understood at the molecular level. Various complementary studies have revealed at least four molecular subgroups of medulloblastoma that harbor distinct genetic alterations, patient demographic indicators, and survival trends (Northcott et al. 2011). Specifically, medulloblastomas of the Wnt subgroup, so named due to the frequent activating mutations in  $\beta$ -catenin and consequent hyperactivation of the Wnt pathway, tend to exhibit the best outcomes of all medulloblastomas. Shh subgroup tumors, which exhibit intermediary survival trends, tend to harbor activating mutations or copy number alterations in Shh pathway regulators such as *SUFU*, *PTCH1*, and *MYCN*. *P53* mutations are also enriched in these tumors (Jones et al. 2012). Group 3 and 4 medulloblastomas comprise tumors with either very poor (Group 3) or intermediate (Group 4) outcomes. As indicated by their arbitrary

names, Group 3 and 4 tumors exhibit relatively few recurrent genetic alterations in well-defined signaling pathways, with a major distinction between these tumors being frequent copy number gain and overexpression of *MYC* in Group 3 tumors (Northcott et al. 2011; Robinson et al. 2012). Although *MYC*, *MYCN*, and *OTX2* genetic alterations have been described in Group 3 and 4 medulloblastomas (Northcott et al. 2012; Robinson et al. 2012), additional recurrent genetic alterations in these subgroups remain enigmatic. Although evidence is accumulating for frequent alterations of chromatin modifier genes such as *CHD7*, *EZH2*, *MLL2/3*, and *UTX* across the medulloblastoma subgroups (Jones et al. 2012; Pugh et al. 2012; Robinson et al. 2012), the functional consequences of these alterations and the mechanistic relationships among them remain unknown.

Our studies have focused on *OTX2* copy number gain and overexpression in medulloblastoma, which was first identified in our lab using digital karyotyping (Di et al. 2005), and then validated and functionally characterized in subsequent studies [(Adamson et al. 2010), to which this chapter refers]. The frequency of *OTX2* alterations in Group 3 and 4 medulloblastomas and the ubiquitous nature of its overexpression in non-Shh medulloblastomas (Adamson et al. 2010; Northcott et al. 2011) indicates it is functionally relevant to a broad spectrum of these tumors. In these studies, we have assessed *OTX2* genetic alterations in medulloblastoma and functionally characterized this oncogene using standard *in vitro* and tumorigenicity assays.

## **2.2 Methods**

### **2.2.1 Medulloblastoma samples**

Primary medulloblastomas were obtained from the Duke University Medical Center Brain Tumor Biorepository and the Hospital for Sick Children, University of Toronto, in accordance with Internal Review Board (IRB)-approved protocols. Fresh-frozen samples (n=201) and medulloblastoma cell lines (n=11) were processed at the Centre for Applied Genomics at the Hospital for Sick Children for 100K or 500K Affymetrix Genechip SNP (Single Nucleotide Polymorphism) array analysis as previously described (Northcott et al. 2009b). For gene expression analysis (n=103), RNA was processed and hybridized to Affymetrix Genechip Human Exon 1.0 ST Arrays, and then classified using unsupervised hierarchical clustering as described in (Northcott et al. 2009a; Northcott et al. 2011).

### **2.2.2 Cell culture, transfections, and xenografts**

Medulloblastoma cells were cultured in 1× Improved Modified Eagle's Zinc Option Medium (MEM-ZO) supplemented with 10% fetal bovine serum (FBS), 1% HEPES, and 0.22% NaHCO<sub>3</sub>. All transfections were carried out with Lipofectamine 2000 according to the manufacturer's instructions. For Western blotting, cells were plated at a density of  $2 \times 10^5$  cells/well in 6-well plates and harvested 48h following transfection. For MTT assays, cells were plated at  $10^5$  cells/well in 24-well plates, and then assessed for viability at the indicated times based on reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-

diphenyltetrazolium bromide. For retinoic acid experiments, cells were treated with 13-*cis* retinoic acid (Sigma) for the indicated times and then assessed for viability and OTX2 mRNA level using the MTT assay and reverse transcriptase (RT) followed by quantitative PCR (qPCR), respectively. RT was carried out on 1 µg of total RNA using the BioRad iScript kit, and cDNA was amplified in an ABI 7900 HT Fast Real-Time PCR System using Kapa reagents and SYBR green detection.

For xenograft tumorigenicity assays,  $10^7$  medulloblastoma cells were transfected with pSUPER vectors harboring either scramble or OTX2 shRNA sequences, and then stereotactically injected into the cortical hemisphere of athymic nude mice. Animals were then monitored for symptoms of brain tumor formation and sacrificed when symptomatic. Brains were fixed overnight in neutral buffered formalin, paraffin embedded, and then sectioned.

RK3E rat kidney epithelial cells were maintained in DMEM supplemented with 10% FBS. Stable OTX2-expressing cell lines were generated by transfection with pEGFP-N1 or pEGFP-OTX2, selection with 500 µg/ml Geneticin for 4-5 weeks, and then plating at clonal density to establish monoclonal cell lines expressing GFP-tagged OTX2 or GFP alone. For colony formation assays,  $5 \times 10^3$  cells were plated onto 24-well plates, and the number of colonies per plate was determined after cells were allowed to grow for two weeks. For xenograft tumorigenicity assays,  $10^6$  cells were stereotactically injected into the cortical hemisphere of nude mice and the mice were sacrificed 18 days following

transplantation for harvesting of brains and subsequent histological analysis. Brains were processed as described above.

### **2.2.3 Retrovirus production and infection**

pBABE retroviruses were generated as described in (O'Hayer and Counter 2006). Briefly, HEK293T cells were transfected on consecutive days with 3 µg of pCL10A1 helper plasmid and 3 µg of pBABE viral cargo plasmid (either empty or containing OTX2 cDNA) using Fugene 6. Viral supernatants were collected 24 and 48 hours following final transfection and stored at -80 °C. Supernatants were thawed and added to D425 medulloblastoma cells, which were selected with 25 µg/mL Geneticin for 6 days prior to experiments.

### **2.2.4 Western blotting and immunohistochemistry**

Sections of paraffin-embedded brains were stained for Ki67 (Lab Vision) or biotinylated OTX2 (R & D) using standard immunohistochemical techniques. For Western blotting, cell lysates were suspended in Laemmli loading buffer supplemented with β-mercaptoethanol and resolved on a 4-12% SDS-PAGE gel (Invitrogen). Proteins were transferred to PVDF membranes and probed for OTX2 (R & D), MYC (Santa Cruz), and GAPDH (Santa Cruz) using standard techniques.

### **2.2.5 CHIP-chip and expression analysis of OTX2 siRNA-treated cells**

We performed chromatin immunoprecipitation (ChIP) experiments with an anti-OTX2 antibody (R & D) using the ChIP Assay Kit from Upstate Cell Signaling.

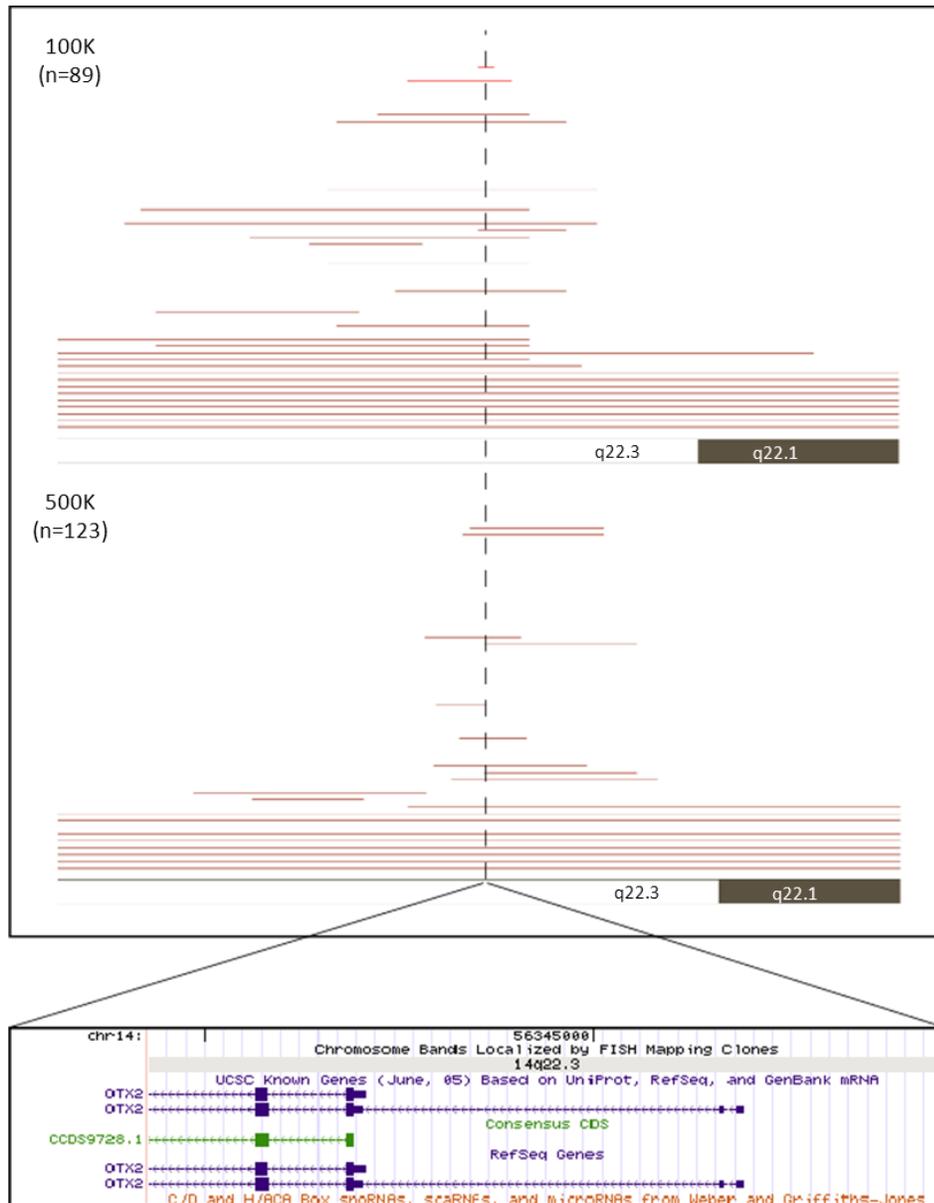
Immunoprecipitated DNA from the enriched and control input DNA samples were labeled, pooled and co-hybridized to NimbleGen HG18 Promoter Tiling microarrays, representing the entire known human promoter sequences tiled at 100-bp resolution across the promoter regions 3.5kb upstream to 0.75kb downstream of all known transcriptional start sites. Because each probe has an associated genomic coordinate, one can plot the intensities as a function of chromosome locations and then reconstruct the enrichment of particular DNA fragments compared to the genomic background. The enriched regions appear as peaks representing protein-bound DNA fragments. We used a model-based analysis of two-color arrays (MA2C) algorithm (Song et al. 2007) to analyze the ChIP-chip data. Resultant gene lists were functionally categorized using GOstat (Beissbarth and Speed 2004).

For expression analysis, D425 medulloblastoma cells were transfected with either scramble or OTX2 siRNAs and then harvested 44 hours later for RNA purification. RNA was then processed and hybridized to Affymetrix HG-U133A Plus 2.0 arrays for gene expression profiling. Hybridization, washing, staining, scanning, and data analysis were performed at the Duke Microarray Core Facility. Expression levels were analyzed using GeneSpring GX version 7.3.1.

### ***2.3 Prevalence and subtype specificity of OTX2 copy number gain in medulloblastoma***

In collaboration with the University of Toronto, we assessed copy number alterations (n=212) and expression status (n=103) of *OTX2* in a large cohort of

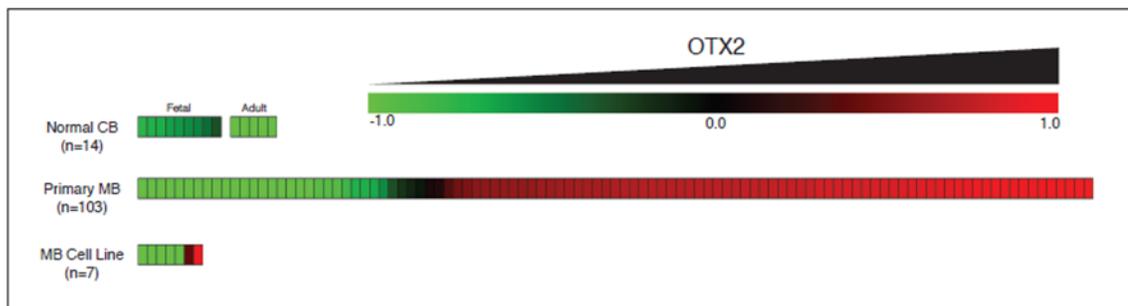
medulloblastomas using quantitative single nucleotide polymorphism (SNP) arrays and gene expression microarrays, respectively. Of tumors subjected to microarray analysis, each was classified into one of four molecular subgroups (Northcott et al. 2011). Among all tumors analyzed for copy number alterations, *OTX2* was the most frequent target of focal copy number gain, with 21% of tumors (n=44 of 212 samples) exhibiting genomic gain of the minimal region encompassing the *OTX2* gene (Figure 1).



**Figure 1: Copy number gains of chromosome 14 targeting *OTX2* in medulloblastoma**

Above, medulloblastoma samples comprise each row (n=212, including 201 primary medulloblastomas and 11 medulloblastoma cell lines), and copy number gains as determined by Affymetrix SNP arrays are indicated in red, with low level gains represented by light lines and high level gains represented by dark lines. Below, UCSC Genome Browser snapshot exhibiting the *OTX2* gene being completely included in the region of overlap for this copy number gain

Supporting a functional role for OTX2 in medulloblastoma pathogenesis, expression of this gene as measured by microarray analysis indicated that in addition to copy number gain, OTX2 is overexpressed in medulloblastoma relative to normal cerebellum in the majority of these tumors (~74%, n=76 of 103 primary medulloblastoma samples; Figure 2).

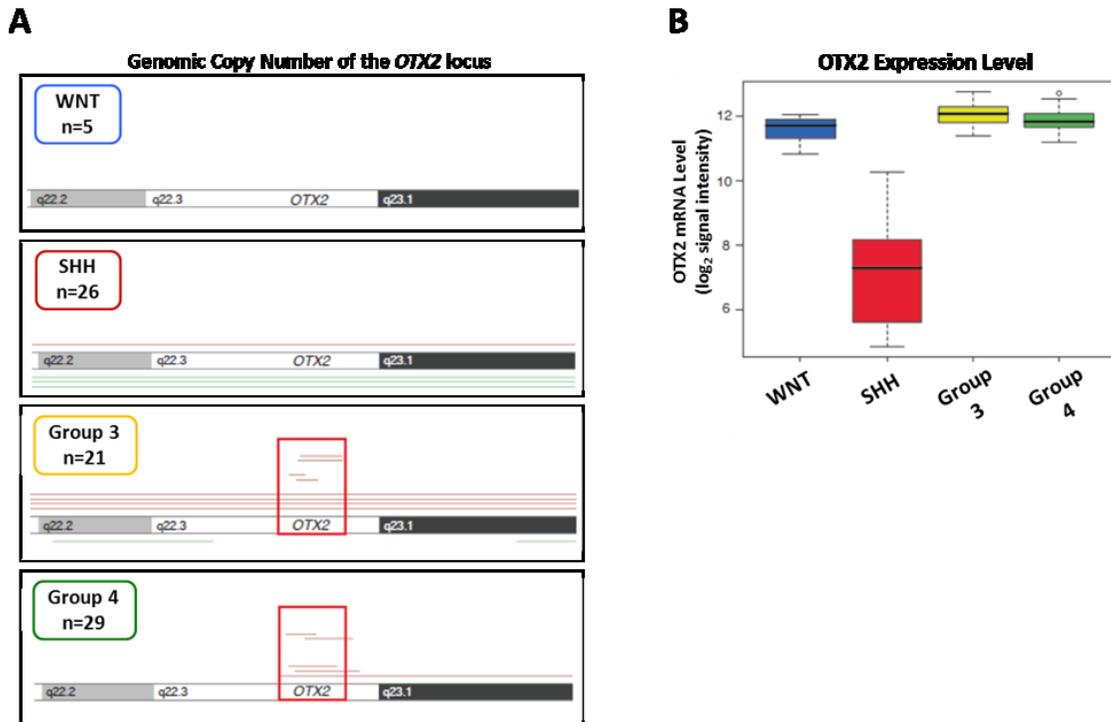


**Figure 2: Frequent OTX2 expression in medulloblastoma**

**OTX2 mRNA levels in normal fetal and adult cerebellum (CB) samples, primary medulloblastomas, and medulloblastoma cell lines.**

Of molecularly classified medulloblastomas, *OTX2* gains were exclusively found in Group 3 and 4 tumors (Figure 3), whereas Group 3 tumors exhibited the most frequent *OTX2* gains, present in 38% of these samples (n=8 of 21 samples). Microarray analysis revealed a bimodal distribution of *OTX2* mRNA levels among medulloblastomas (Figures 2 and 3), with the Shh subgroup expressing the lowest levels (near or below that of normal cerebellum) and the non-Shh subgroups (e.g. Wnt, Group 3, and Group 4) expressing high levels of *OTX2* relative to the Shh group tumors (Figure

3) or normal cerebellum (Figure 2). These findings reveal potential roles for OTX2 in a variety of phenotypically distinct medulloblastomas.



**Figure 3: Subgroup-specific OTX2 copy number gains and mRNA expression**

(A) Copy number alterations at the OTX2 locus in medulloblastoma subtypes. Red indicates copy number gain and green indicates loss. (B) OTX2 mRNA level, as determined by microarray analysis, in medulloblastoma subtypes.

## 2.4 Requirement for OTX2 in tumor maintenance

### 2.4.1 OTX2 is required for medulloblastoma proliferation *in vitro*

To determine the role of OTX2 in established medulloblastomas, we tested the requirement for OTX2 in the proliferation of medulloblastoma cells *in vitro*. To this end,

we knocked down OTX2 in a panel of medulloblastoma cell lines using a variety of siRNA and shRNA's and then measured cell viability using MTT assays (Figure 4). These experiments demonstrated that over a long timecourse, OTX2 inhibition reduced medulloblastoma cell proliferation *in vitro* (Figure 4). Similar results obtained from multiple siRNA and shRNA sequences relative to scramble siRNA's and shRNA's revealed that this effect is specific to OTX2 knockdown and limited to OTX2-expressing cell lines.

#### **2.4.2 OTX2 is required for xenograft tumorigenicity**

If OTX2 is indeed critical for the maintenance of medulloblastomas *in vivo*, it represents an attractive target for selective therapeutics against this tumor. To test this possibility, we stably transfected medulloblastoma cells with OTX2-targeted shRNA (Figure 5A), and then injected pooled cells into the cortex of immunodeficient *nu/nu* mice. OTX2 knockdown indeed prolonged survival of recipient animals ( $p = 0.0061$ , log-rank test) (Figure 5B-C). Immunohistochemistry of residual tumors from animals injected with OTX2 shRNA-treated tumor cells revealed that these tumors still expressed OTX2 (data not shown), confirming that residual tumors had escaped OTX2 knockdown and further supporting an absolute requirement for OTX2 in tumor growth *in vivo*.

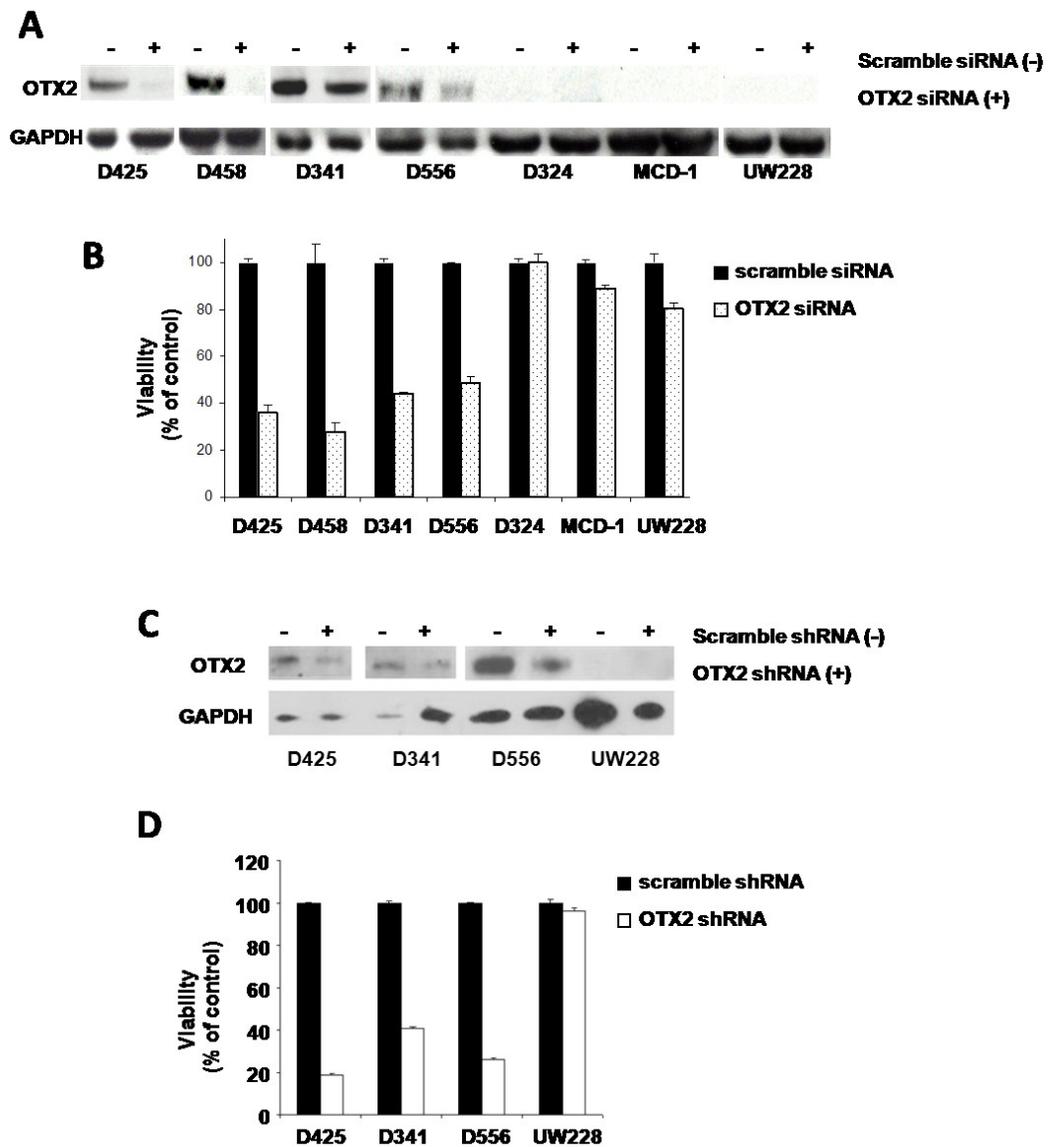
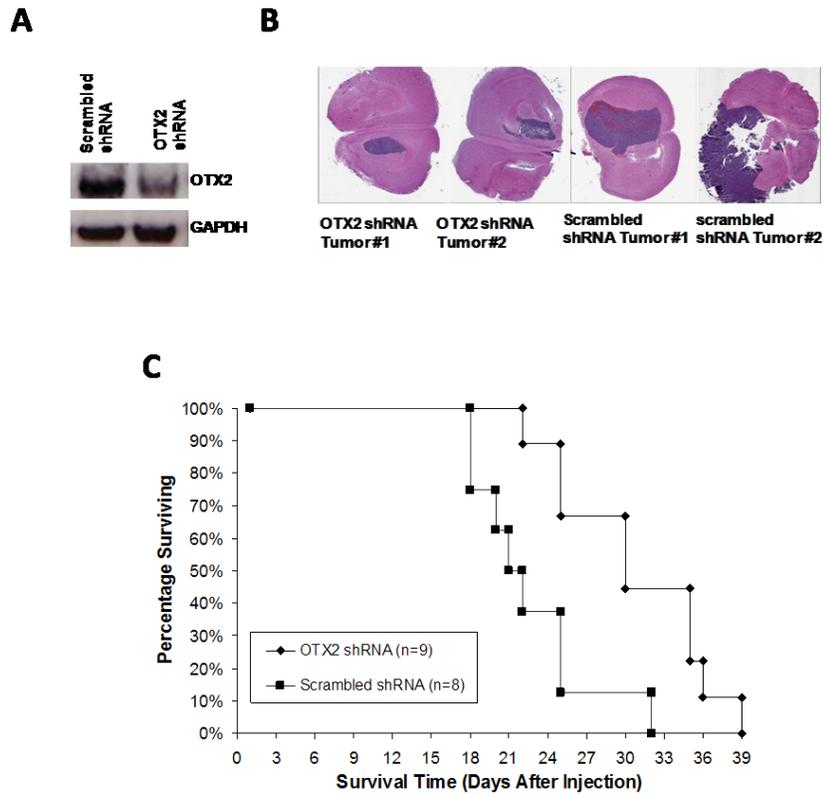


Figure 4: OTX2 is required for medulloblastoma cell proliferation

(A) Medulloblastoma cells were treated with either scramble or OTX2 siRNA and then assayed for OTX2 protein expression by Western blotting. (B) Medulloblastoma cells treated as in (A) were assessed for viability after three doubling times using the MTT assay. (C) Medulloblastoma cells were treated with either scramble or OTX2 shRNA and then assayed for OTX2 protein expression as in (A). (D) Medulloblastoma cells treated as in (B) were assessed for viability using the MTT assay.



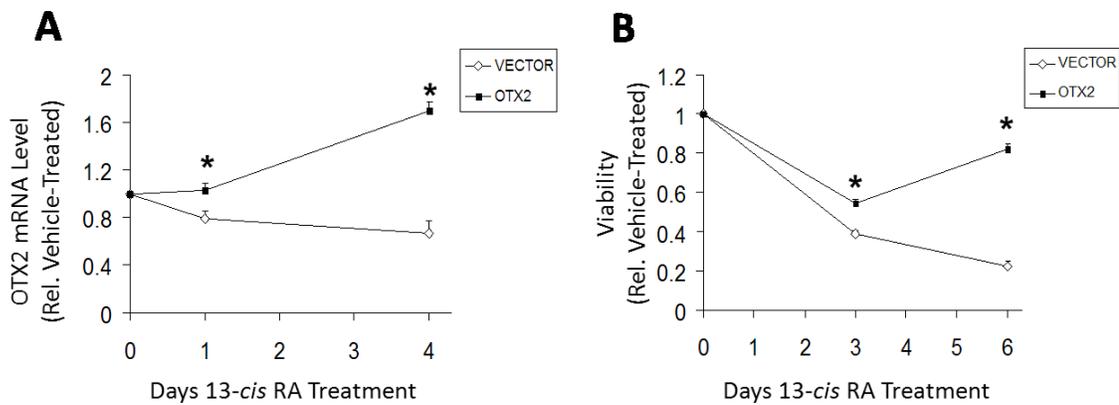
**Figure 5: OTX2 is required for medulloblastoma xenograft tumorigenicity**

(A) Medulloblastoma cells were treated with either scramble or OTX2 shRNA and then assayed for OTX2 protein expression by Western blotting. (B) Medulloblastoma cells treated as in (A) were injected intracranially and animals were monitored for symptoms of tumor formation. Representative H & E stained brain sections are shown. (C) Survival of mice injected with either OTX2 or scramble shRNA-treated medulloblastoma cells.

### 2.4.3 OTX2 silencing is a critical event in retinoic acid-induced growth suppression

Retinoids are known differentiation agents for medulloblastoma (Hallahan et al. 2003; reviewed in: Wortham and Yan 2009). Although our lab had previously demonstrated that retinoids downregulate OTX2 (Di et al. 2005), a direct role for OTX2

repression in retinoid-mediated growth suppression of medulloblastoma had not been demonstrated. To test this possibility, OTX2-expressing medulloblastoma cells were infected with pBABE viruses containing cDNAs for OTX2 expressed from the viral LTR. This ectopic promoter prevents OTX2 downregulation by retinoids (Figure 6). Following retinoid treatment, OTX2 virus-transduced cells were rescued from the growth inhibitory effects of retinoids observed in empty virus-transduced cells. These results support a critical role for OTX2 repression in the effect of retinoids upon medulloblastoma, which is supported by independent experiments published elsewhere (Bai et al. 2010).

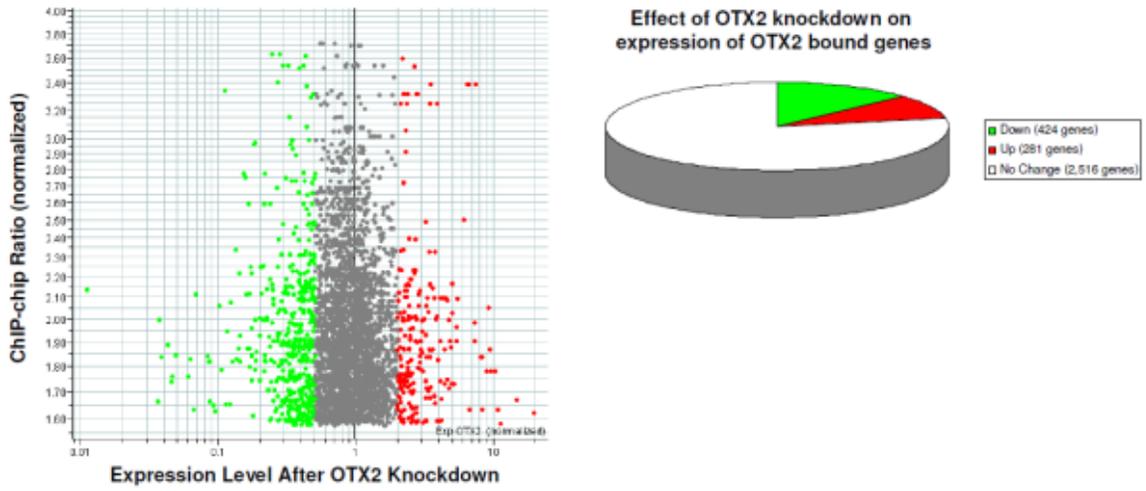


**Figure 6: OTX2 repression is required for the effect of retinoids upon medulloblastoma proliferation**

(A) Medulloblastoma cells were infected with either OTX2 or control viruses, and OTX2 mRNA level (endogenous and ectopic) was assessed using RT-qPCR. (B) Medulloblastoma cells treated as in (A) were grown in the presence of 7  $\mu$ M 13-*cis* retinoic acid (13*cis*RA) or vehicle and then assessed for viability with the MTT assay. \* $p < 0.05$ , Student's t-test.

## ***2.5 Identification and classification of OTX2 transcriptional targets***

As a transcription factor, OTX2 exerts its function by binding to DNA and regulating target genes (Adamson et al. 2010). An understanding of gene networks regulated by OTX2 would identify the cellular processes coordinated by this oncogene in medulloblastoma cells. To identify OTX2 target genes we have applied ChIP-chip technology, which pairs chromatin immunoprecipitation (ChIP) with high-density oligonucleotide tiling microarrays (chip), using an anti-OTX2 antibody to recognize OTX2-associated chromatin in D425 human medulloblastoma cells. A set of enriched promoter regions were identified and their associated genes were compiled into a list of OTX2-bound genes. To identify genes actively regulated by OTX2, we applied microarray analysis to D425 medulloblastoma cells following OTX2 knockdown. Differentially expressed genes (>2-fold change) were compiled into a list and then compared with the list of genes bound by OTX2, resulting in the identification of 424 OTX2-activated genes and 281 OTX2-repressed genes (Figure 7).



**Figure 7: OTX2 regulates hundreds of genes in medulloblastoma**

**Left, OTX2 target genes were identified by promoter ChIP-on-chip for DNA immunoprecipitated using an anti-OTX2 antibody in D425 cells, and regulated genes were identified by comparing gene expression levels between cells treated with either OTX2 or scramble siRNA. Right, summary of the direction of regulation by OTX2 at its target genes.**

To reveal the cellular processes coordinated by OTX2 target genes, the final sets of OTX2-activated or -repressed genes (e.g. those bound and regulated by OTX2) were analyzed for enrichment of gene ontologies using GOSTat (Beissbarth and Speed 2004). Of genes directly activated by OTX2, there was a highly significant overrepresentation of those regulating cell cycle and proliferation (34 genes,  $p < 10^{-9}$ , and 31 genes,  $p < 10^{-9}$ , respectively) (Table 1). Of genes silenced by OTX2, those involved in nervous system development (27 genes,  $p < 10^{-15}$ ) and regulation of transcription (68 genes,  $p < 10^{-10}$ ) were overrepresented (Table 2). These findings are in general agreement with studies

published elsewhere (Bunt et al. 2010; Bunt et al. 2011a). Notably, OTX2 was also found to regulate *CD133*, *MYC*, and *PTEN*, which are central genes relevant to neural stem cell and cancer stem cell identification and maintenance (Fulfs et al. 2002; Singh et al. 2004; Zheng et al. 2008; Pei et al. 2012). Additionally, we found that sustained MYC expression is required for the maintenance of medulloblastoma *in vitro* (Adamson et al. 2010), suggesting that OTX2 serves to maintain these tumors in part through transactivation of *MYC*. We have also compared the list of genes bound by OTX2 (3,221 genes total) with the top 100 genes identified in (Pomeroy et al. 2002) whose expression most clearly distinguishes medulloblastoma from normal cerebellum. This comparison revealed that while OTX2 binds regulatory regions of ~13% of promoters, these genes are substantially enriched for those distinguishing medulloblastoma from normal cerebellum, with OTX2 binding 34 such genes ( $p < 0.0001$ , chi-squared test). Thus, OTX2 likely plays a major role in establishing the molecular signature of medulloblastoma.

**Table 1: Functional categories of genes transcriptionally upregulated by OTX2 in medulloblastoma**

<u>Parent Gene Ontogeny</u> Representative Child Ontogenies	<b>OTX2 Targets</b> <b>Gene Count (%)</b>	<b>Human Genome</b> <b>Gene Count (%)</b>	<b>p-value</b>
<b><u>Cellular Process</u></b>	297 (70)	19591 (58)	2.12E-05
Cell cycle	34 (8)	839 (3)	1.93E-10
Cell proliferation	31 (7)	745 (2)	4.67E-10
Apoptosis	25 (6)	855 (3)	4.40E-04
<b><u>Localization</u></b>	90 (21)	4481 (13)	1.62E-04
Cell motility	11 (3)	383 (1)	4.65E-02
<b><u>Metabolic Process</u></b>	225 (53)	14566 (43)	5.60E-04
Protein metabolic process	95 (22)	5858 (17)	3.11E-02
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	93 (22)	5848 (17)	4.74E-02
<b><u>Establishment of Localization</u></b>	79 (19)	4135 (12)	2.67E-03
Protein transport	22 (5)	866 (3)	1.32E-02
<b><u>Locomotion</u></b>	5(1)	73 (0.2)	5.17E-03
Regulation of cell migration	4(1)	60 (0.2)	1.18E-02
<b><u>Developmental Process</u></b>	62(15)	3347 (10)	2.80E-02
Cell differentiation	42(10)	1810 (5)	1.15E-03

**Table 2: Functional categories of genes transcriptionally repressed by OTX2 in medulloblastoma**

<u>Parent Gene Ontology</u>	<b>OTX2 Targets</b>	<b>Human Genome</b>	<b>p-value</b>
Representative Child Ontogenies	<b>Gene Count (%)</b>	<b>Gene Count (%)</b>	
<b><u>Developmental Process</u></b>	72 (26)	3347 (10)	2.71E-16
Cell differentiation	40 (14)	1810 (5)	1.83E-09
Nervous system development	27 (10)	716 (2)	6.57E-16
Programmed cell death	18 (6)	862 (3)	1.03E-03
<b><u>Biological Regulation</u></b>	114 (41)	6731 (20)	2.88E-16
Regulation of metabolic process	74 (26)	4150 (12)	2.05E-11
Regulation of signal transduction	15 (5)	800 (2)	1.52E-02
<b><u>Cellular Process</u></b>	217 (77)	19591 (58)	2.08E-09
Cell communication	72 (26)	5560 (16)	6.81E-04
Cellular component organization and biogenesis	55 (20)	3277 (10)	9.03E-07
Establishment and/or maintenance of chromatin architecture	15 (5)	450 (1)	7.89E-05
<b><u>Multicellular Organismal Process</u></b>	61 (22)	3822 (11.25)	2.00E-06
<b><u>Gene Expression</u></b>	75 (27)	5454 (16)	3.10E-06
Regulation of transcription	68 (24)	3654 (11)	1.71E-11
<b><u>Metabolic Process</u></b>	151 (54)	14566 (43)	2.40E-03
Regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	69 (25)	3713 (11)	2.07E-11
<b><u>Rhythmic Process</u></b>	4 (1)	74 (0.2)	5.02E-03

## **2.6 Evidence of a role for OTX2 in tumorigenesis**

Considering that OTX2 is expressed in a broad spectrum of medulloblastomas, and that OTX2 can be broadly expressed within tumors, it is possible that OTX2 plays a

role in transformation. To assess this possibility, we tested the effects of OTX2 expression upon RK3E cells, which are E1A-immortalized rat kidney epithelial cells commonly used to assess the contribution of genes to transformation. RK3E cells stably transfected with an OTX2 expression construct exhibited enhanced colony formation potential *in vitro* (Figure 8A-B) and when injected into the cortical hemisphere of nude mice, OTX2-expressing cells gave rise to proliferative, vascular tumors (Figure 8C), whereas vector-transfected cells generally formed scanty proliferative foci ( $p < 0.001$ , Student's t-test for Ki67 indices, Table 3).

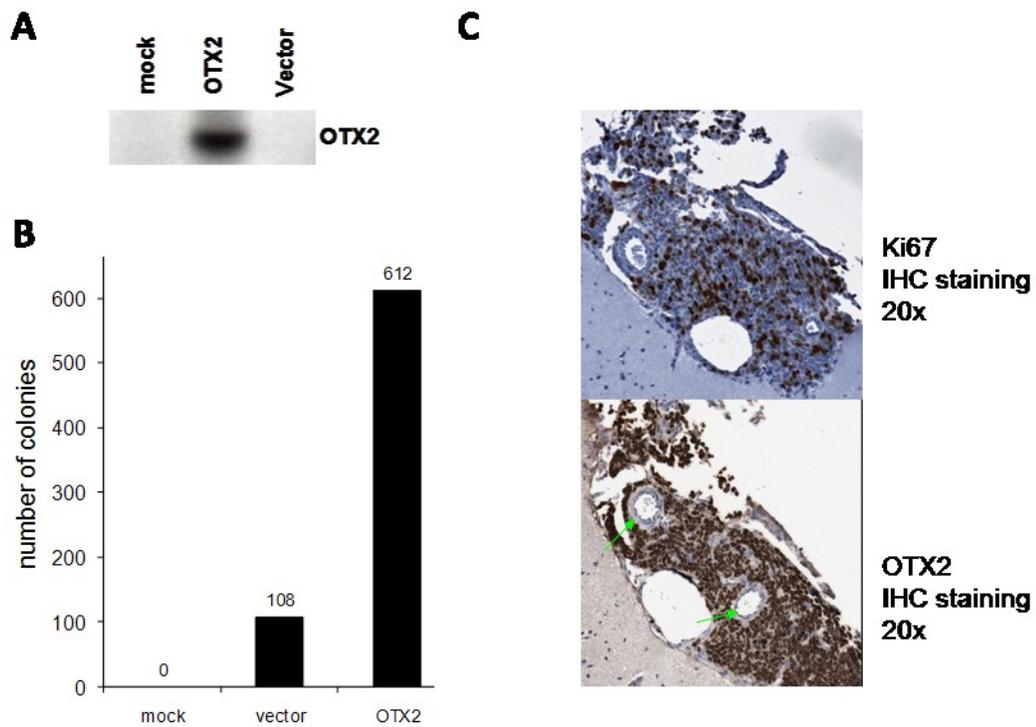


Figure 8 (continued): OTX2 enhances tumorigenicity of immortalized RK3E cells

(A) Stable OTX2-expressing RK3E cell lines were established and then assessed for OTX2 protein expression using Western blot. (B) OTX2-expressing and control RK3E cells were assessed for clonal proliferation using the colony formation assay. (C) Cell lines from (A) were intracranially injected into the cerebral hemispheres of nude mice. Brains were harvested 18 days later for H & E staining and immunohistochemistry.

Table 3: Tumor formation rate and Ki67 indices of tumors and foci derived from RK3E intracranial xenografts.

<b>ID</b>	<b>Tumor Formation</b>	<b>Ki67 Average Score (%)</b>
OTX2-1	Y	12.25
OTX2-2	Y	12.9
OTX2-3	Y	10
OTX2-4	Y	12
OTX2-5	Y	10.4
OTX2-6	Y	12.9
OTX2-7	Y	10.495
OTX2-8	Y	9.085
VEC-1	Y	9.67
VEC-2	Y	5.255
VEC-3	Y	3
VEC-4	N	NA
VEC-5	N	NA
VEC-6	N	NA
VEC-7	N	NA

Frequency of intracranial tumor formation incidence and tumor Ki67 scoring in mice injected with RK3E cells stably transfected with pEGFP-N1 (VEC-N) or pEGFP-OTX2 (OTX2-N). Each line represents a single mouse.

### **3. Identifying the mechanisms of OTX2 overexpression in medulloblastoma**

#### ***3.1 Introduction: the transcriptional dysregulation of oncogenes and tumor suppressors as key events in tumorigenesis***

While genetic alterations affecting gene sequence and/or structure are classical mechanisms contributing to tumorigenesis, there is also a longstanding appreciation that functions of “driver” genes can be modulated by essence of transcriptional dysregulation and subsequent overexpression or repression (Nusse and Varmus 1982; Shen-Ong et al. 1982; He et al. 1998). This mechanism is underscored by accumulating data implicating alterations of the general epigenetic machinery in medulloblastoma pathogenesis (Northcott et al. 2009b; Robinson et al. 2012). Similarly, alterations in broad epigenetic signatures such as polycomb-mediated trimethylation of histone 3 lysine 27 have been described in these tumors (Robinson et al. 2012). The inheritability of epigenetic marks is consistent with the model of clonal expansion of a tumor precursor lesion—like genetic alterations, such alterations would be passed on to the progeny of clonally-expanding preneoplastic cells. In addition to their roles in the formation of a tumor, mechanisms of transcriptional dysregulation have potential to be exploited therapeutically (Delmore et al. 2011).

We have previously described a critical and widespread role for sustained OTX2 expression in medulloblastoma tumor maintenance (Adamson et al. 2010). Similarly, there is some evidence that OTX2 could play some role in transformation of normal

hindbrain cells (Wortham et al. 2012), suggesting that OTX2 could play diverse roles in both the establishment and progression of these tumors. As such, understanding the mechanisms whereby OTX2 is dysregulated would be informative of both tumor pathogenesis and to the design of selective therapeutics targeting this critical gene. As previously described, a subset of medulloblastomas (~21% of tumors) overexpress OTX2 by essence of increased gene dosage; however, there remains a substantial proportion of medulloblastomas (~50%) that lack genetic alterations of *OTX2* yet overexpress this gene (Adamson et al. 2010). Thus, for a large subset of tumors, the mechanisms of OTX2 overexpression remain unknown, and we have sought to better understand the transcriptional regulation of OTX2 in these tumors.

Findings from large scale efforts to map transcriptional regulatory elements have revealed that *cis*-acting regulators mediating gene expression are dynamic entities whose location is best determined by identifying loci exhibiting particular epigenetic characteristics and assessing these elements for functional activity (Crawford et al. 2006; Visel et al. 2009; Ernst et al. 2011; Haeussler and Joly 2011; Song et al. 2011; Thurman et al. 2012). These elements are frequently cell type-specific, may be far removed from the regulated gene's transcriptional start site, and are not necessarily highly-conserved, thus the identification of transcriptional regulatory elements demands advanced approaches. To this end, we have utilized an emerging technology, DNase-seq, which takes advantage of the observation that the endonuclease DNase I preferentially cleaves DNA

that is associated with non-nucleosomal proteins, which bind less tightly to DNA and typically serve regulatory roles (Crawford et al. 2006). Mapping of cut sites preferentially targeted by DNase reveals various transcriptional regulatory elements including promoters, enhancers, insulators, repressors, and locus control regions (Packer et al. 1991; Crawford et al. 2004), underscoring that DNase hypersensitivity (and, by extension, chromatin accessibility) is frequently an indicator of some regulatory function. With this approach, it is possible to identify *cis*-acting elements that are poorly-conserved, distal to their target genes, active in particular cellular contexts, and of broad functional classes. Importantly, this assay can reveal regulatory element utilization in different cellular states; as such, we have characterized chromatin accessibility patterns in medulloblastoma cells harboring various levels of OTX2 expression.

## **3.2 Methods**

### **3.2.1 Cell culture, transfections, and gene expression studies**

Medulloblastoma cells were maintained as described above. For luciferase assays, 24 well plates were seeded with  $10^5$  cells per well, and then cells were transfected with 1.3  $\mu$ g of reporter plasmid and 67 ng of pRL-CMV internal control plasmid using Lipofectamine 2000. 24 hours later, cells were harvested and luciferase activity was measured using the Promega Dual-Luciferase Reporter Assay System. For knockdown experiments, cells were transfected with 100  $\mu$ M final concentrations of the following siRNA's: Scramble: GAGUCAACCUUAUGAUACUtt, OTX2 #1

GGAGGUGGCACUGAAAAUCtt, OTX2 #2 GGACACUAAUUCAUCUGUAAtt, CRX #1: GCUCACAGGUCCUAGUGAUtt and CRX #2: GUGUGGAUCUGAUGCACCAtt.

Reporter plasmid insertion sequences are described as Hg19 genomic coordinates in Figure 23. For gene expression experiments,  $5 \times 10^5$  cells were plated onto 6 cm dishes and transfected with 4  $\mu$ g of expression plasmid or siRNAs (at a final concentration of 100  $\mu$ M), and 24 hours later RNA or lysate was prepared for measuring mRNA (by RT-qPCR) or protein levels (by Western blotting). RT-qPCR and Western blotting were performed as described in Chapter 2.2. Chromatin immunoprecipitation was performed with an anti-OTX2 antibody using the ChIP Assay Kit from Upstate Cell Signaling. qPCR was performed on immunoprecipitated DNA, and amplicons of interest were normalized to Line 1 amplicons as a loading control. *All-trans* retinoic acid, *13-cis* retinoic acid, and cycloheximide were obtained from Sigma, and 5-aza-2'deoxyctidine was purchased from MP Biomedicals.

DNA methylation levels were determined in a cohort of medulloblastoma samples using bisulfite sequencing. Briefly, 500 ng of DNA from each sample was bisulfite treated, and 50 ng of treated or untreated DNA was then amplified using Faststar Taq. DNASTAR Seqman Software was used to score each site for methylation state. For DNA demethylation experiments, cells were treated with 1 or 5  $\mu$ M 5-aza-2'deoxyctidine for 5 days before cells were harvested for RT and semi-quantitative PCR.

### 3.2.2 DNase hypersensitive (DHS) site mapping

Medulloblastoma cell nuclei were prepared from  $1-5 \times 10^7$  cells using NP40 concentrations optimized for each cell line to permit complete lysis while avoiding nuclear aggregation. Nuclei were then aliquotted and treated with increasing levels of DNase I enzyme (4 to 24 units per 200 uL reaction) and incubated at 37 °C for 16 minutes. The reaction was stopped with EDTA and then incubated overnight at 37 °C. DNA was then extracted with phenol/chloroform.

DNase-treated DNA was prepared and analyzed as described in (Song et al. 2011). Briefly, digested DNA was blunt-ended, ligated to biotinylated linkers containing an MmeI digestion site (which cuts downstream of its recognition sequence), digested with MmeI, ligated again to linkers at the MmeI cut end, and finally PCR amplified for subsequent analysis with Solexa sequencing. Sequence reads were aligned to the genome and then smoothed using F-seq (Boyle et al. 2008).

For DNase-PCR, digested DNA was quantitated using a UV spectrophotometer and diluted to 6 ng/uL. DNA was amplified with primers of interest using 9 ng of DNA in a 20 uL reaction and quantified relative to a standard curve run for each amplicon representing a range of 10-100% of undigested DNA. For each sample and DNase concentration, amplicons of interest were compared to a known DNase-resistant region as a reference to input amount and to adjust for nonspecific DNase digestion.

### **3.2.3 Motif discovery**

To determine the DNA-binding proteins interacting directly with DHS 4 Fragment A, this 13 bp genomic fragment was extended by 6 bp in either direction to yield a 25 bp sequence TGTCTCCGGGATTAATTATGGGCAC. This sequence was then scanned for transcription factor binding sites using TRANSFAC TFblast (Matys et al. 2006), JASPAR CORE (Bryne et al. 2008), JASPAR HOMEO, and Consite (Sandelin et al. 2004). Motifs identified by protein-DNA interaction in (Hu et al. 2009) were aligned using MapDraw. Google searches were performed using a 5 bp sliding window at every position of the 25 bp query fragment.

## ***3.3 Mapping the chromatin accessibility landscape of the OTX2 locus***

### **3.3.1 Establishing a genomewide chromatin accessibility profile in two OTX2-expressing cell lines**

Very few adult tissues and established cell lines express OTX2 (Boon et al. 2002); as such, to map the regulatory elements mediating OTX2 expression in medulloblastoma with no prior expectations of the location and organization of such elements, we performed DNase-seq to understand the regulatory landscape of this gene in the appropriate cellular context. Nuclei from two OTX2-expressing cell lines, D341 and D721, were treated with DNase and then digested DNA was processed for Solexa sequencing. These cell lines both lack detectable *OTX2* copy number alterations (Boon et al. 2005; Northcott et al. 2011) and represent a range of OTX2 expression levels (D341:

13.1-fold overexpression of OTX2; D721: 90.62-fold overexpression of OTX2; both relative to normal cerebellum as determined by SAGE analysis). Mapping of frequently-digested sequences revealed seven consistent DNase-hypersensitive regions in these two medulloblastoma cell lines (Figure 9 and 10A). We noted that the canonical insulator protein CTCF bound to regions flanking medulloblastoma DHS sites 1-7, suggesting that the regulatory activity for OTX2 transcription likely resides in this region (Figure 10B). Comparison of the chromatin accessibility landscape of medulloblastoma with the rare other cell lines that express this gene (Figure 10A) revealed some overlap between an OTX2-expressing retinoblastoma cell line (Weri-Rb) and minimal overlap with embryonic stem (ES) cells, which express OTX2 from the short isoform promoter [Figure 10C and (Fossat et al. 2005)]. These results indicate that we have revealed both novel and previously-identified regions of open chromatin flanking *OTX2*.

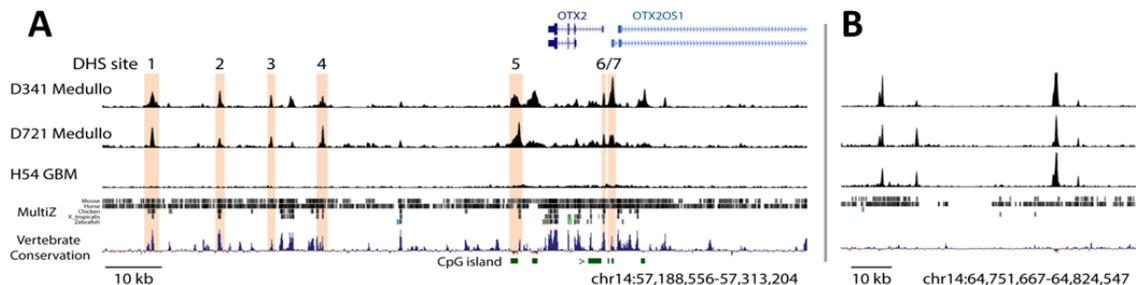
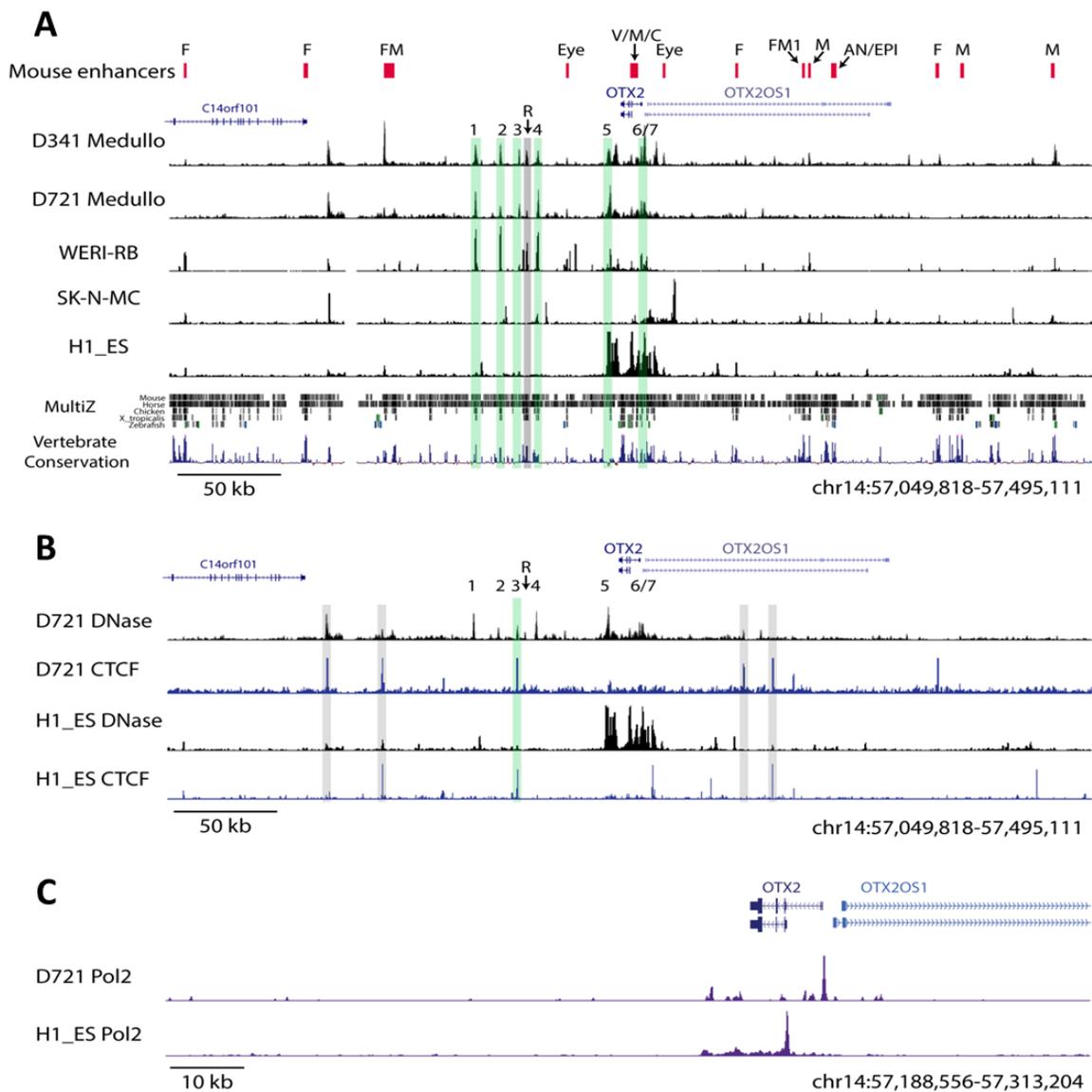


Figure 9 (continued): Chromatin accessibility landscape of medulloblastoma.

UCSC Genome Browser snapshots of the following tracks, from top to bottom: RefSeq genes, DNase-seq tracks for: D341 medulloblastoma (Medullo) cells, D721 medulloblastoma cells, and H54 glioblastoma (GBM) cells, MultiZ vertebrate alignments, PhyloP vertebrate basewise conservation, CpG islands.

(A) Chromatin accessibility flanking the *OTX2* locus. Orange bars indicate consistent and robust DNase hypersensitive (DHS) sites in medulloblastoma.

(B) Chromatin accessibility at a far distal region demonstrating shared DHS sites among all three cell lines. Carat indicates CpG island assessed with bisulfite sequencing in Figure 20.



**Figure 10 (continued): Chromatin accessibility and other structural characteristics of the *OTX2* locus in various *OTX2*-expressing cell types.**

(A) Chromatin accessibility of the *OTX2* locus in a panel of *OTX2*-expressing cell types. From top to bottom, rows indicate: known *OTX2* developmental enhancers labeled with their site of activity, RefSeq genes, DNase-seq tracks for: D341 medulloblastoma (Medullo) cells, D721 medulloblastoma cells, Weri-Rb retinoblastoma cells, SK-N-MC neuroblastoma cells, and H1 embryonic stem (ES) cells, MultiZ vertebrate alignments, PhyloP vertebrate basewise conservation. Red bars indicate known developmental *OTX2* enhancers, green bars indicate medulloblastoma DHS sites, and gray bars indicate overlap with the retina and retinoblastoma regulatory element, DHS "R". (B) DNase-seq tracks overlaid with ChIP-seq reads for the canonical insulator element CTCF in D721 medulloblastoma cells and H1 ES cells. Grey bars indicate CTCF-bound regions and the green bar indicates CTCF binding at medulloblastoma DHS 3. (C) ChIP-seq for Pol2 indicative of distinct transcriptional start sites between medulloblastoma and ES cells. V/M/C, Anterior visceral endoderm, anterior mesendoderm, cephalic neural crest; F, forebrain; M, midbrain; AN/EPI, anterior neuroectoderm/epiblast.

### **3.3.2 Assessing DHS site utilization across medulloblastoma cell lines using a qPCR-based assay of chromatin accessibility**

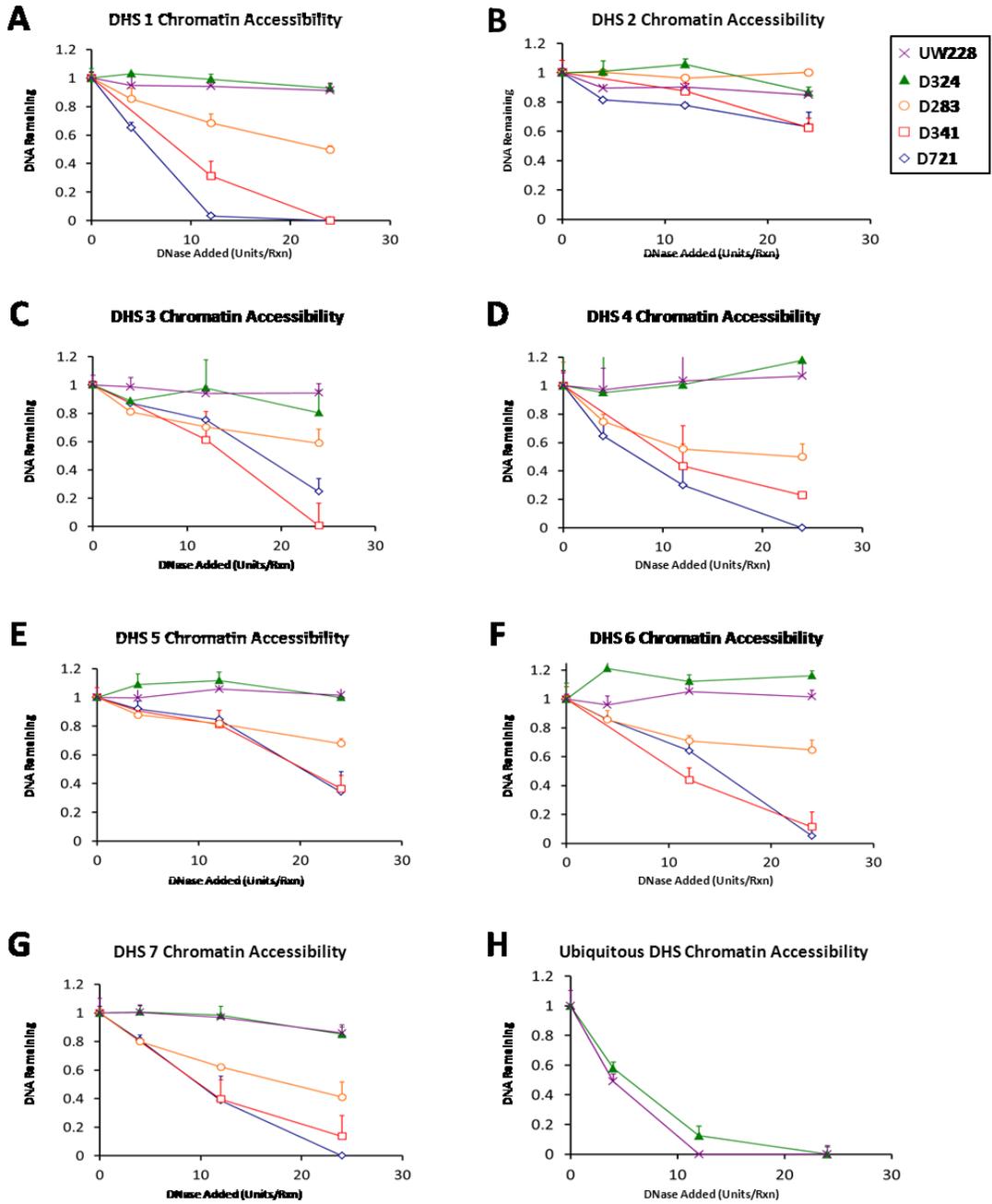
Because regions of open chromatin associated with *OTX2* expression in medulloblastoma, we sought to determine if this association could be extended to medulloblastomas that express high or low levels of *OTX2*. In order to determine DNase hypersensitivity of specific chromatin fragments across samples, nuclei of various medulloblastoma cell lines were treated with DNase, and then qPCR was used to measure the proportion of DNA that was cleaved such that digested DNA is not amplified while DNase-resistant DNA is easily detectable (Figure 11). Detection of a positive control DHS site validates that this approach can detect dynamic levels of genomic DNA following DNase treatment and thus uncover regions of DNase

hypersensitivity. DNase-qPCR confirmed the association between open chromatin at six of the seven DHS sites detected in OTX2-expressing medulloblastoma cells, such that cell lines not expressing OTX2 harbored inaccessible chromatin at these regions (Figure 11). This result is compatible with a model in which differential chromatin accessibility plays a role in dictating OTX2 expression level in medulloblastoma, such that OTX2 expression in medulloblastoma is modulated in part by differential accessibility of DNA to *trans*-acting proteins. Note that DHS 2 did not exhibit substantial DNase hypersensitivity as indicated by qPCR, suggesting that this site is be either a sequencing artifact or rather is not detected with the qPCR approach.

### ***3.4 Assessment of DHS sites for transcriptional regulatory activity***

While DNase-seq identifies the location and differential utilization of potential transcriptional regulatory elements, this assay does not address the nature of regulatory activity of these elements. To determine the role of these elements in modulating transcription, DNA sequences harboring ~500bp of each element were cloned into luciferase reporter vectors to facilitate the measurement of transcriptional output driven by each fragment. To assess the ability of proximal elements to serve as transcriptional promoters (which by definition are located within 2kb of the transcriptional start site), DHS sites 6 and 7 were cloned into a promoterless luciferase reporter vector harboring a generic enhancer from the SV40 virus (pGL3-enh). To assess the enhancer or repressor

activities of DHS sites, each element was cloned into a luciferase reporter vector driven by a minimal, generic promoter from the SV40 virus (pGL3-pro).



**Figure 11 (continued): Chromatin accessibility of medulloblastoma DHS sites across various OTX2-expressing and -nonexpressing cell lines.**

**Nuclei were harvested and treated with increasing concentrations of DNase, and then the proportion of DNA remaining at each site was determined by qPCR. Open and filled markers indicate OTX2-expressing and non-expressing cell lines, respectively.**

Initial luciferase assays revealed promoter activity for both DHS sites 6 and 7 and enhancer activities for DHS sites 1, 4, and 7 (Figure 12A-B). To determine the specificity of these activities to OTX2-expressing cell lines, luciferase assays were performed for each of the active DHS sites in a panel of OTX2-expressing and nonexpressing medulloblastomas (Figure 12C-D). Notably, the promoter activities of DHS sites 6 and 7 were not consistently active in OTX2-expressing cell lines tested, and DHS sites 1 and 7 exhibited promoter activities in all cell lines tested (e.g. were not specific to OTX2-expressing cell lines). These differences may reflect the variable genetic backgrounds of these cell types and suggest that activity of DHS sites 1 and 7 are not likely to be the cause of OTX2 expression in medulloblastoma. On the other hand, DHS 4 exhibited consistent and specific enhancer activity in OTX2-expressing cell lines, indicating that differential enhancer activity of this DHS site (likely due to differential activity of its cognate *trans*-acting factor) may underlay differential OTX2 expression in medulloblastoma.

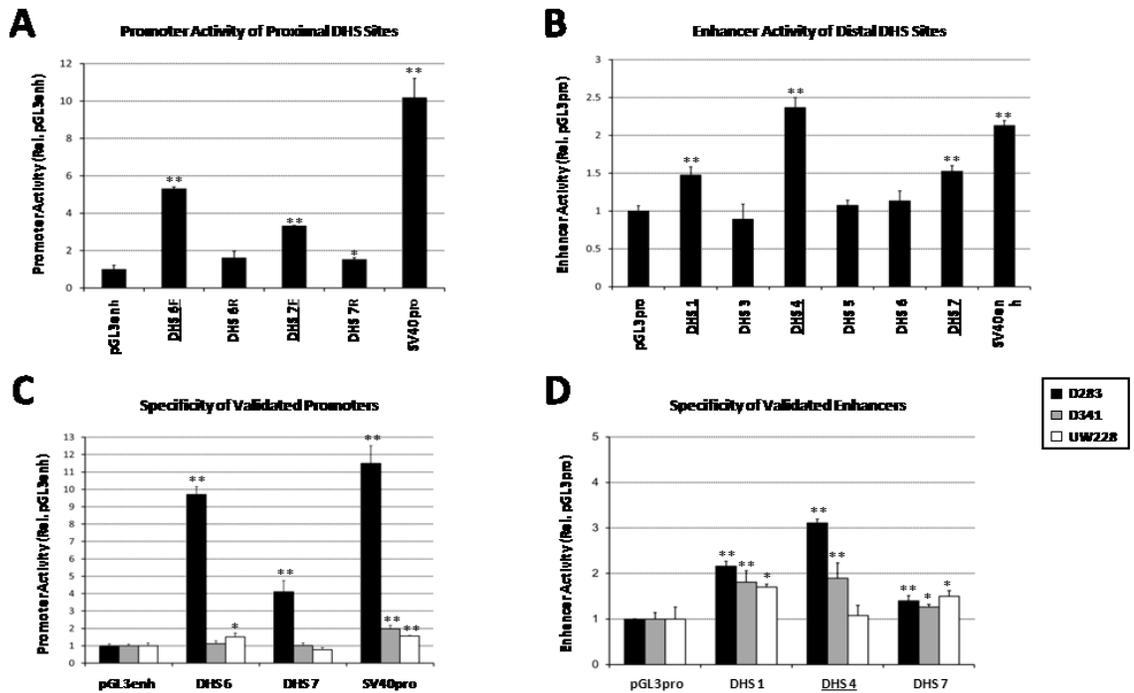


Figure 12: Transcriptional activity of medulloblastoma DHS sites.

(A) Promoter activity of *OTX2*-proximal DHS sites were assayed by determining their ability to drive expression of a luciferase reporter gene in cooperation with a generic enhancer in D283 medulloblastoma cells. (B) Enhancer activity of all medulloblastoma DHS sites were assayed by determining their ability to drive expression of a luciferase reporter gene driven by a minimal, generic promoter in D283 medulloblastoma cells. (C) Specificity of promoter activity as determined by transcriptional activity in a panel of *OTX2*-expressing (D283, D341) and non-expressing (UW228) medulloblastoma cell lines. \* $p < 0.05$  relative to empty vector, \*\* $p < 0.01$  relative to empty vector, Student's t-test. Error bars indicate standard deviation.

### **3.5 Identification of an insulator element repressing DHS 1**

A standard indication of the spatial limits of a transcriptional regulatory module is the presence of insulator elements as implicated by the presence of the canonical insulator protein CTCF. In a parallel project identifying binding sites of various transcription factors in a collection of diverse cell types (Lee et al. 2012), ChIP-seq for CTCF was carried out in D721 medulloblastoma cells. This assay identified CTCF binding both far downstream of the DHS sites investigated above as well as at DHS 3 (Figure 10B). To validate the insulator activity of this element, which in its current organization could potentially affect the ability of DHS site 1 to enhance *OTX2* expression, we constructed a luciferase reporter vector comprised of the following elements: DHS 1, DHS 3, a minimal promoter from the SV40 virus, and finally the luciferase cDNA (Figure 13). In this configuration we are able to determine the ability of DHS 3 to interfere with the enhancer activity of DHS 1. Accordingly, we found that DHS 3 indeed represses the ability of DHS 1 to serve as an enhancer when placed in the configuration observed in native chromatin (Figure 13). This result suggests that DHS 1 may not interact with the *OTX2* promoter in its natural organization in the genome.

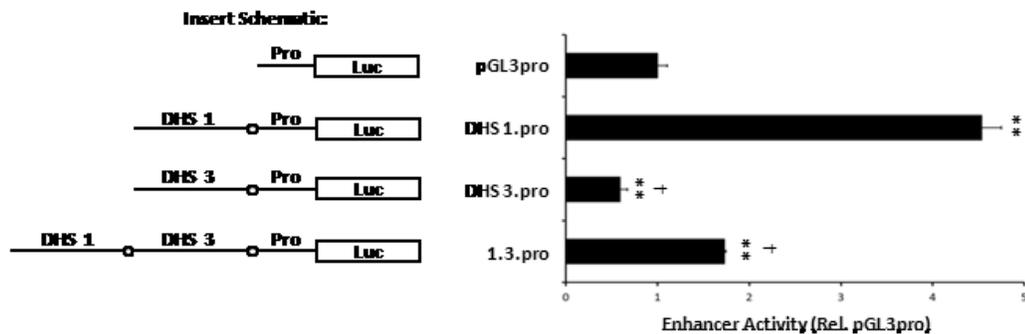


Figure 13: Insulator activity of DHS 3

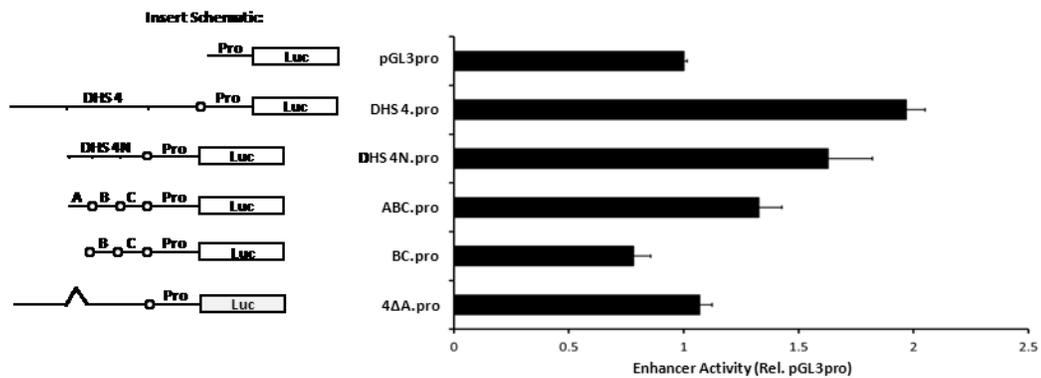
The ability of DHS 3 to insulate the enhancer activity of DHS 1 was assessed using luciferase assays with the depicted constructs. \* $p < 0.05$  relative to empty vector, \*\* $p < 0.01$  relative to empty vector, † $p < 0.05$  relative to DHS 1.pro, Student's t-test. Error bars indicate standard deviation.

### 3.6 Characterization of trans-acting factors mediating enhancer activity of DHS 4

#### 3.6.1 Identification of a minimally-required region of the DHS 4 enhancer

The finding that DHS 4 exhibited specific activity in OTX2-expressing medulloblastomas suggests that this differential activity could dictate the inherent differences of OTX2 transcription in medulloblastoma. As such, we sought to determine the *trans*-acting factors that may coordinate this differential activity, with the goal of identifying upstream regulators of OTX2 in this tumor. To this end, we progressively consolidated the minimal regions required for enhancer activity of DHS 4 so as to optimize the results from a scan for defined transcription factor binding sequences. The ~500bp fragment was first reduced to a central 70bp fragment, which retained most of its

enhancer activity (Figure 14). Reconstruction of this fragment using linkers (ligated at restriction fragment sites and labeled A-C) facilitated the stepwise deletion of sequence elements within this 70bp region. Deletion of Fragment A from the 70bp DHS 4 construct completely abolished DHS 4 enhancer activity, indicating that this 13bp fragment is absolutely critical for the activity of this DHS site.



**Figure 14: Identifying the minimally-required fragment for DHS 4 activity**

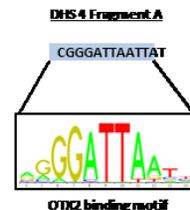
Enhancer activity for progressive deletions of DHS 4 was determined with the luciferase assay using the constructs depicted. Error bars indicate standard deviation.

### 3.6.2 Scanning for protein binding motifs in the minimally-required region of DHS 4

The minimally-required 13bp fragment of DHS 4 was first extended by 6bp in either direction to ensure that informative sequence information was not lost. The resulting 25bp sequence was then scanned for transcription factor binding sites using various publically-available resources (Figure 15) generated 7 high-confidence

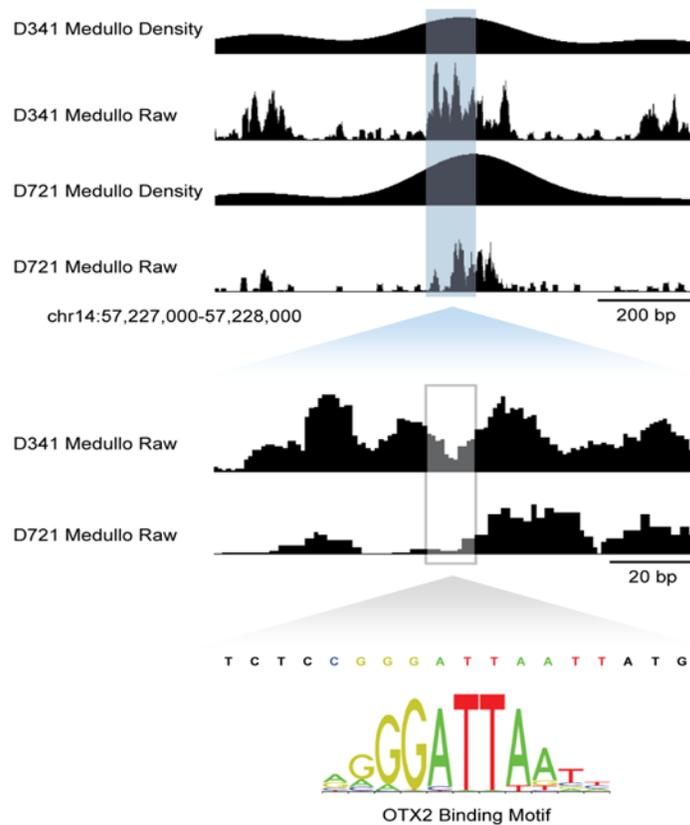
transcription factor binding site predictions (Sandelin et al. 2004; Matys et al. 2006; Bryne et al. 2008; Hu et al. 2009). Manual inspection of SAGE expression data for these candidate regulators revealed that few of these factors were expressed in medulloblastoma as expected for a *trans*-acting factor positively influencing OTX2 transcription. However, the OTX2 protein itself bound to the 13bp fragment A region with very high confidence ( $p = 0.0001$ ), suggestive of an autoregulatory loop. Additionally, a DNase footprint (Neph et al. 2012) could be identified at the putative OTX2 binding site within DHS 4 in medulloblastoma cells (Figure 16).

Database	Rationale	# TF's represented	# Hits	# Expressed in Medulloblastoma
JASPAR CORE	Curated database of vertebrate motifs	130	8	2
JASPAR HOME0	Curated database of homeobox motifs	176	17	2
TRANSFAC TFblast	Curated database of vertebrate motifs	398	0	
Consite	Integrates conservation scores	130	0	
Manual alignment to motifs in Hu et al.	Confirmed <i>in vitro</i> interactions	201	8	1
Google Search	ID otherwise undocumented motifs	?	9	2



**Figure 15: Identifying *trans*-acting regulators binding to Fragment A, the minimally-required sequence of DHS 4**

**Left, results of various database searches for binding motifs in the Fragment A sequence. Right, highly-scoring match for the OTX2 binding motif comprising 11bp of DHS 4 Fragment A.**



**Figure 16: Identifying a DNase footprint at the OTX2 binding motif in DHS 4**

**Smoothened (Density) and unsmoothened (Raw) DNase tracks for medulloblastoma cell lines are shown at increasing resolution. Oscillating patterns of DNase hypersensitivity are observed flanking the sequence corresponding to DHS 4 Fragment A (grey box), which contains the OTX2 binding motif.**

Treatment of medulloblastoma cells with OTX2-targeted siRNA's verified that OTX2 is indeed required for the enhancer activity of DHS 4 (Figure 17), validating that OTX2 regulates this distal enhancer whose activity distinctly associates with OTX2 expression in medulloblastoma.

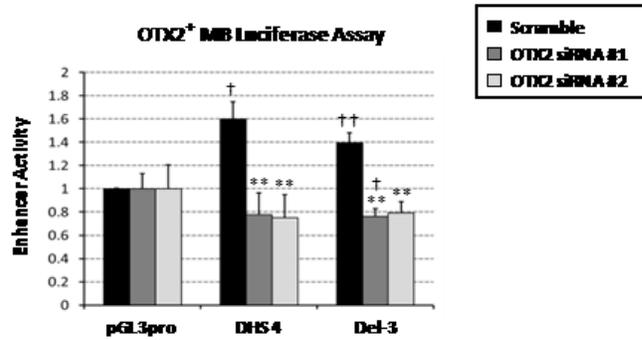


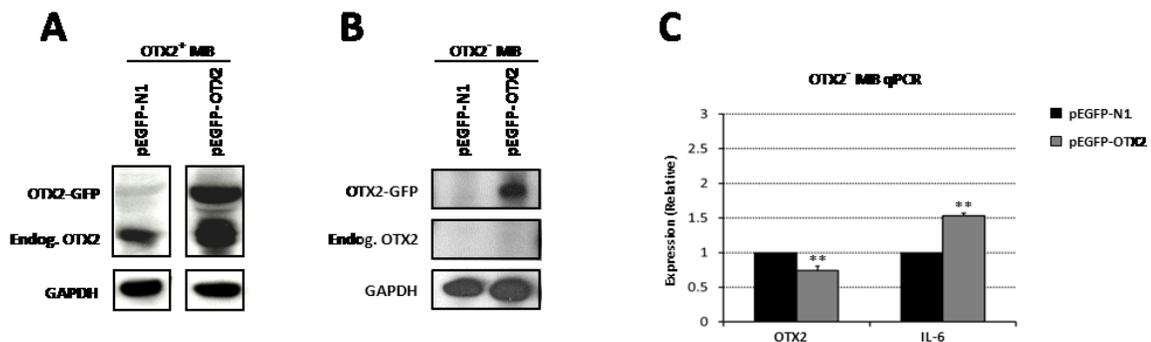
Figure 17: Assessing the requirement of OTX2 for DHS 4 enhancer activity

Luciferase assays were performed with medulloblastoma cells transfected with the indicated plasmids. \* $p < 0.05$  relative to scramble siRNA, \*\* $p < 0.01$  relative to scramble siRNA, † $p < 0.05$  relative to pGL3pro, †† $p < 0.01$  relative to pGL3pro, Student's t-test. Error bars indicate standard deviation.

### 3.6.3 Validation of an OTX2 autoregulatory loop in a distal transcriptional enhancer

To determine whether OTX2 can mediate its own expression in the context of native chromatin, we tested the ability of ectopic OTX2 to enhance or initiate transcription from the endogenous *OTX2* gene. Critically, this experiment measures the ability of OTX2 to modulate its own expression in the context of native chromatin, which may be additionally modified and exhibit complex interactions that are not modeled with luciferase reporter assays. To this end, we expressed an EGFP-tagged OTX2 gene in medulloblastoma cells and measured the expression level of endogenous OTX2 protein, which migrates more quickly through a PAGE gel used for protein separation prior to Western blotting. Expressing ectopic OTX2 in medulloblastoma cells that naturally express OTX2 indeed enhanced protein expression of endogenous OTX2 (Figure 18A), suggesting that OTX2 can indeed regulate its own transcription in the context of its

native genomic locus. On the other hand, expressing ectopic OTX2 in medulloblastoma cells that do not normally express this gene did not initiate expression of endogenous OTX2 (Figure 18B-C). Notably, ectopic OTX2 activated the known OTX2 target gene IL-6 [Figure 18C; (Bunt et al. 2010)] in cells that don't naturally express OTX2, indicating that the ectopic protein is functional in these cells. This result suggests that in this context, the OTX2 gene is refractory to transcriptional activation by *trans*-acting factors. This result is consistent with our previous observations that chromatin accessibility of medulloblastoma DHS sites (including DHS 4) is distinct between OTX2-expressing and non-expressing cell lines (Figure 11). Thus it is possible that repressive chromatin is responsible for the failure of OTX2 to initiate its own expression in this cellular context.



**Figure 18: Assessing the ability of OTX2 to enhance its own expression**

Medulloblastoma cells naturally expressing OTX2 (A) or not expressing OTX2 (B) were transfected with the indicated plasmids, and expression level of endogenous and ectopic (GFP-tagged) OTX2 was assessed by Western blot. (C) Assessment of mRNA level of the OTX2 target gene IL-6 by RT-qPCR in cells treated as in (B). \* $p < 0.05$  relative to empty vector, \*\* $p < 0.01$  relative to empty vector, Student's t-test. Error bars indicate standard deviation.

Finally, to confirm that endogenous OTX2 associates with DHS 4 (e.g. in the absence of overexpression), chromatin immunoprecipitation for endogenous OTX2 was performed. Indeed, endogenous OTX2 was found to associate with DHS 4; this interaction was not observed with a control fragment or in cell lines that do not express OTX2 (Figure 19).

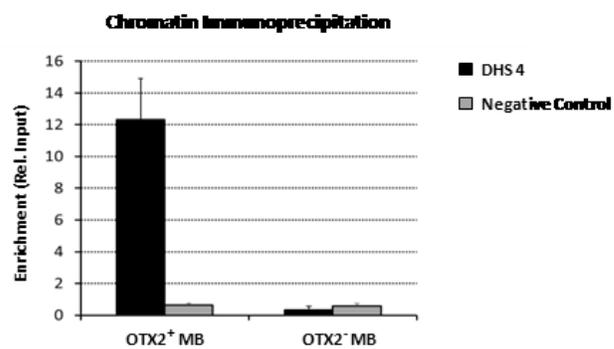


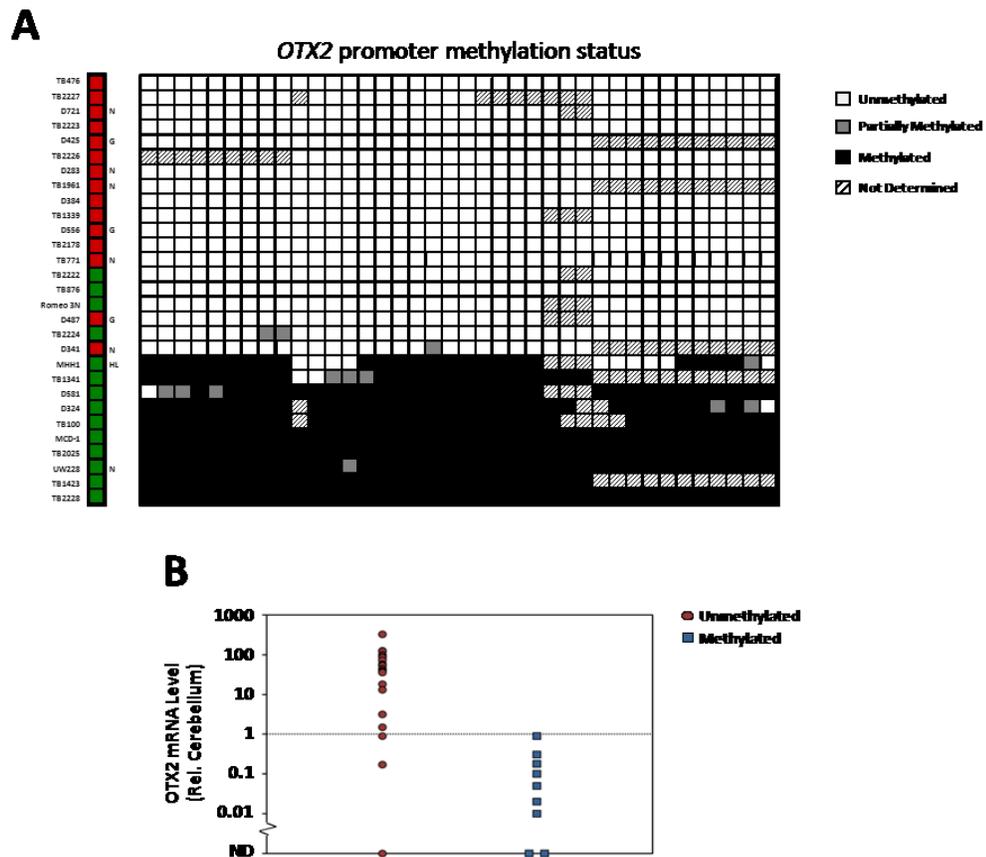
Figure 19: Assessing binding of endogenous OTX2 to DHS 4

Medulloblastoma cells naturally expressing OTX2 were harvested for chromatin immunoprecipitation with an anti-OTX2 antibody and fragment enrichment was then assessed using qPCR. Error bars indicate standard deviation.

### ***3.7 Characterization of DNA methylation patterns associated with OTX2 silencing***

An additional potential level of OTX2 transcriptional regulation is that of promoter DNA methylation, as indicated by the presence of CpG islands found in the OTX2 promoter region (Figure 9). To assess this possibility, bisulfite sequencing was performed on DNA purified from a collection of medulloblastoma samples (n=33). Levels of cytosine methylation of the CpG island located immediately downstream of the OTX2 transcriptional start site were suggestive of a binary promoter methylation

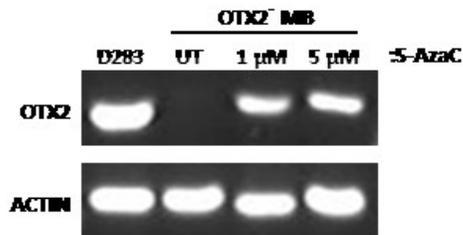
status among medulloblastomas (Figure 20A), which is consistent with DNA methylation patterns observed in other cell lines and tumor types. With this in mind, the *OTX2* promoter can be classified as predominately harboring either methylated or unmethylated CpG's. Comparison of *OTX2* expression levels between medulloblastoma samples exhibiting different promoter methylation states reveals a strong association between *OTX2* promoter methylation and transcriptional repression (Figure 20B). Although promoter methylation appears to be sufficient to repress *OTX2*, not all tumors exhibiting an unmethylated promoter expressed *OTX2*, indicating that positively-acting factors in addition to a permissive chromatin state is required for *OTX2* expression.



**Figure 20 (continued): Assessing the methylation status of the *OTX2* promoter in medulloblastoma**

**(A) Leftmost column indicates *OTX2* expression status (red: overexpressed, green: not expressed) and *OTX2* copy number status for each sample, if known (G: gain, N: normal, HL: heterozygous loss). Chart indicates methylation status of CpG sites in columns and medulloblastoma samples in rows for representative samples and sequence reads.**

To validate that *OTX2* promoter methylation is of functional consequence, *OTX2*-nonexpressing medulloblastoma cells were treated with 5-aza-2'deoxyctidine, which was shown to partially rescue *OTX2* expression (Figure 21), thus indicating that *OTX2* promoter methylation actively represses *OTX2* in medulloblastoma.



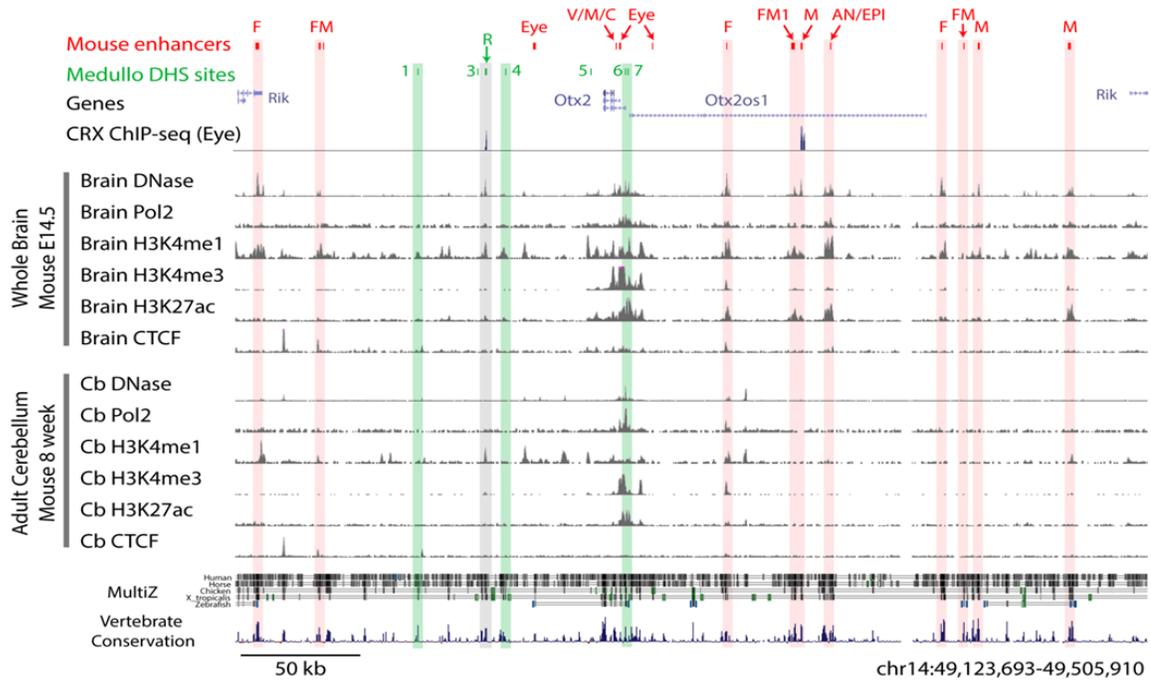
**Figure 21: Promoter methylation actively represses *OTX2* expression**

***OTX2*-nonexpressing medulloblastoma cells were treated with the indicated concentrations of 5-aza-2'deoxyctidine (5-AzaC) for 5 days and then assessed for *OTX2* mRNA expression with RT followed by semiquantitative PCR. D283 cell line cDNA was used as a positive control for *OTX2* expression.**

### **3.8 Characterization of the epigenetic modifications of relevant OTX2 regulatory elements during embryonic brain development**

Having characterized the transcriptional regulatory elements that mediate differential OTX2 expression in medulloblastoma, we then asked if these elements are implemented in normal development of the embryonic brain. Alternatively, these elements may represent novel regulatory elements in the context of medulloblastoma (e.g. due to exceptionally high levels of some *trans*-acting regulator that is never observed during normal development or physiology). To this end, we first mapped the medulloblastoma DHS sites onto the mouse genome (Figure 22) to facilitate comparison of these sites with their homologous regions during normal brain development at a timepoint (e.g. embryonic day 14.5) when OTX2 is dynamically expressed (Fossat et al. 2006). Additionally, we mapped known OTX2 developmental enhancers (Kimura-Yoshida et al. 2004; Kurokawa et al. 2004a; Kurokawa et al. 2004b; Emerson and Cepko 2011; Muranishi et al. 2011), revealing that medulloblastoma DHS sites have not been previously implicated in OTX2 regulation. Examination of the chromatin states of the embryonic brain reveals that epigenetic marks indicative of active enhancers and promoters (e.g. H3K4me1 and H3K27Ac as well as DNase accessibility) associate with DHS sites that are active in medulloblastoma (DHS sites 4, 6, and 7; Figure 22). As combinatorial assessment of chromatin structure is predictive of functional activity (Ernst et al. 2011), we classified medulloblastoma DHS sites according to these criteria as assessed in the embryonic brain and adult cerebellum (Figure 22 and 23). Strikingly, the

function of most medulloblastoma DHS sites as validated in Figure 12 could be accurately predicted from chromatin status in the embryonic brain or cerebellum.



**Figure 22: Chromatin structure of the *OTX2* locus in the developing brain and adult cerebellum**

UCSC genome browser snapshots of the mouse genome are shown for the following tracks: known *Otx2* developmental enhancers labeled with site of activity, regions of homology to medulloblastoma DHS sites, Refseq genes, CRX ChIP-seq of mouse eye from (Corbo et al. 2010) E14.5 whole mouse brain DNase-seq, Pol2 ChIP-seq, H3K4me1 ChIP-seq, H3K4me3 ChIP-seq, H3K27Ac ChIP-seq, CTCF ChIP-seq, from (Shen et al and Genome Browser), MultiZ vertebrate alignment, PhyloP vertebrate conservation. Green bars indicate regions homologous to medulloblastoma DHS sites exhibiting signatures of active transcriptional regulation, pink bars indicate regions with known enhancer activity in the mouse brain, and the grey bar indicates DHS "R". Enhancer activity locations abbreviated as in Figure 10.

	DHS 1	DHS 2	DHS 3	DHS "R"	DHS 4	DHS 5	DHS 6	DHS 7
Coordinates (hg19, chr14)	57,197,081-57,197,624	57,208,951-57,209,448	57,218,296-57,218,708	57,221,649-57,222,405	57,227,265-57,227,738	57,261,971-57,262,522	57,276,973-57,277,519	57,278,349-57,278,688
Position Rel. TSS (long isoform)	+80,000	+68,000	+59,000	+55,000	+50,000	+15,000	0	-1000
Differential Accessibility?	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Predicted Function*	Enhancer	N.D.	Insulator	Strong Enhancer	Enhancer	Enhancer	Active Promoter	Active Promoter
Actual Function	Enhancer	N.D.	Insulator	N.D.	Enhancer	None Found	Promoter	Promoter/Enhancer
Functional Specificity	No	N.D.	N.D.	N.D.	Yes	N.D.	No	No
Known Status in Human CNS	RB DHS	RB DHS	No hypersensitivity	RB DHS	RB, NB, DHS	RB DHS (weak)	RB DHS	No hypersensitivity
Known Status in Mouse CNS	WB H3K4me1 (weak)	N.D.	No histone marks or DHS <sup>5</sup>	WB & CB H3K4me1, WB H3K27Ac	WB H3K4me1, WB H3K27Ac (weak)	WB & CB H3K4me1, WB H3K27Ac	WB & CB H3K4me3, WB & CB H3K27Ac	WB H3K4me1, WB & CB H3K27Ac
Known TF Binding			CTCF (MB, RB, ES)	CRX (eye)				
Conservation (Alignment)	Xenopus	Xenopus	Mammals	Xenopus	Xenopus	Xenopus	Zebrafish	Mammals
Homology to Ms (% Identity)	94%	94%	86%	90%	92%	90%	90%	94%
Homology to Ms (Location, chr14)	49,200,069-49,200,450	49,212,826-49,213,316	49,225,083-49,225,338	49,228,290-49,228,866	49,236,689-49,236,966	49,272,515-49,272,725	49,287,095-49,287,474	49,288,335-49,288,569
Activity Detected in Mouse <sup>5</sup>	None	None	None	Nasal Cavity**	Nasal Cavity**	Nasal Placode	N.D.	N.D.

Figure 23: Location and characteristics of medulloblastoma DHS sites

"Known Status in Mouse CNS" refers to E14.5 forebrain or adult cerebellum

\*Based on classification system proposed in (Ernst et al. 2011), predicted from histone status (H3K4me1, H3K4me3, H3K27Ac), DNase sensitivity, and CTCF binding in mouse embryonic forebrain or adult cerebellum

§Assessed (at embryonic timepoints only) in: Kimura-Yoshida et al. 2004, Kurokawa et al. 2004a, and Kurokawa et al. 2004b. \*\*DHS 4 and DHS "R" reside in the in same fragment assayed

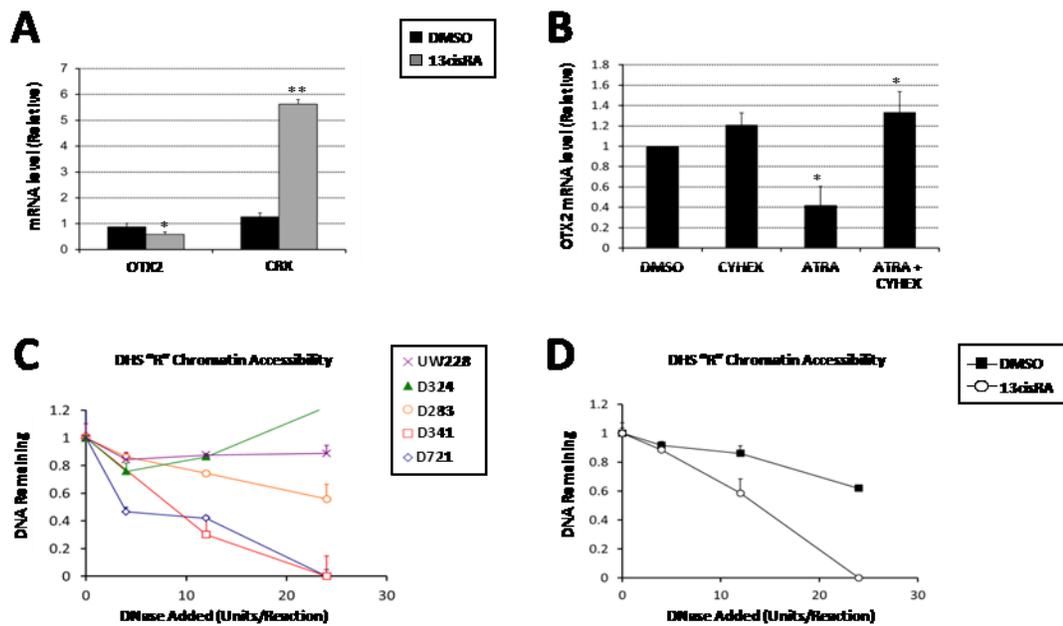
Abbreviations: CNS, central nervous system; Ms, mouse; WB, whole embryonic brain; CB, cerebellum; RB, retinoblastoma; NB, neuroblastoma; MB, medulloblastoma; TF, transcription factor; N.D., not determined.

These observations suggest that some cell type in the developing brain (which in this dataset is comprised of myriad cell types comprising the forebrain, midbrain, and hindbrain) exhibits similar utilization of *OTX2* transcriptional regulatory elements as some medulloblastomas. Accordingly, a cell type of the developing brain that fails to transcriptionally repress *OTX2* may well serve as a cell of origin for *OTX2*-expressing medulloblastomas. Alternatively, it is possible that over the course of transformation, an *OTX2*-nonexpressing cell type assumes the chromatin landscape of some cell type of the embryonic brain that exhibits such epigenetic and chromatin accessibility patterns. Considering our previous observations that in some contexts, the *OTX2* promoter is refractory to the initiation of *OTX2* transcription by *trans*-acting factors, it seems most likely that *OTX2* expression is inherited from an *OTX2*-expressing cell type rather than induced *de novo*.

### **3.9 Identifying the mediator of retinoic acid-induced repression of *OTX2***

Retinoids are known to repress *OTX2* mRNA in the context of normal development as well as in medulloblastoma. While searching for evidence of regulatory transcription factors binding to DHS 4, we found a ChIP-seq dataset implicating the binding of CRX, which binds to a sequence quite similar to the *OTX2* binding site, to a region homologous to a weak medulloblastoma DHS (and strong retinoblastoma DHS) in the developing mouse retina (Figure 22). Given this observation, we designated this site as DHS "R". Considering the regulatory interaction between Crx and Otx2 in the

eye, in which retinoids are highly-concentrated, we sought to determine the role of CRX in retinoid-mediated OTX2 repression in medulloblastoma. CRX is expressed in some medulloblastomas (Boon et al. 2002) and can be further induced with retinoids (Fig 24A). Supporting a role for some translatable intermediate in retinoid-mediated OTX2 repression, cycloheximide (CYHEX) treatment abolishes the repressive effect of retinoids upon OTX2 (Figure 24B). Finally, we found that the putative CRX binding site, DHS "R", is DNase hypersensitive in medulloblastomas and becomes more DNase-accessible upon retinoid treatment (Figure 24D). While retinoid treatment increased most DHS sites somewhat, we found that this effect was most prominent in the distal cluster of DHS sites 1, 3, "R", and 4 (data not shown).



**Figure 24 (continued): Dynamics of CRX expression and its putative binding site during retinoid treatment of medulloblastoma**

mRNA expression levels of OTX2 and CRX as determined by RT-qPCR in medulloblastoma following treatment with 2  $\mu$ M 13-*cis* retinoic acid (13cisRA) for 24 hours. (B) OTX2 mRNA expression, determined as in (B), following treatment with 2  $\mu$ M all-*trans* retinoic acid (ATRA) and/or 35  $\mu$ M cycloheximide (CYHEX) for 8 hours. (C) Basal DNase sensitivity of DHS "R" in a panel of medulloblastoma cell lines, determined as in Figure 11. Open and filled markers indicate OTX2-expressing and non-expressing cell lines, respectively. (D) DNase sensitivity of DHS "R" in medulloblastoma cells treated with 2  $\mu$ M 13-*cis* retinoic acid (13cisRA) or DMSO for 24 hours.

Based on these observations, we sought to identify the requirement for CRX in retinoid-induced repression of OTX2. Treating medulloblastoma cells with siRNA against CRX, we demonstrated inhibition of basal and retinoid-induced CRX mRNA levels using qPCR (Figure 25). Although cells treated with CRX siRNA's exhibited variable basal levels of OTX2, CRX siRNA abrogated OTX2 repression by all-*trans* retinoic acid (Figure 25). Despite this effect upon OTX2 repression, canonical retinoic acid signaling was intact in these cells, as indicated by robust induction of the direct RAR-regulated target gene, RAR $\beta$ . These results are consistent with a model in which retinoids induce CRX expression, which then binds to DHS "R" in medulloblastoma to repress transcription of OTX2. These findings are consistent with our observation that a translated intermediate is required for OTX2 repression by retinoids.

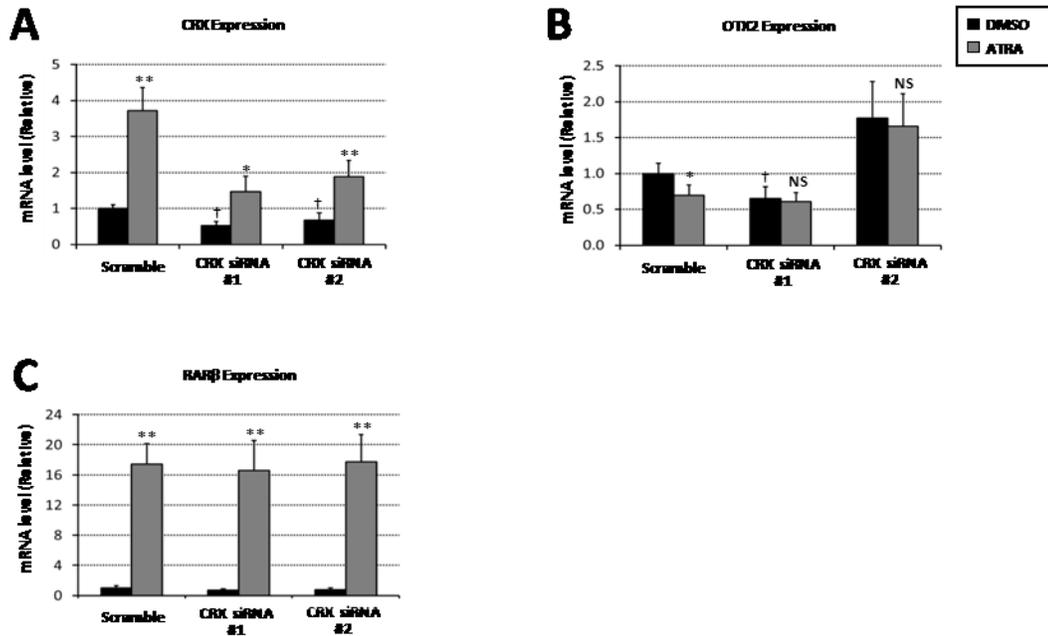


Figure 25: CRX induction is required for retinoid-mediated OTX2 repression

Medulloblastoma cells were treated with either scramble or CRX siRNA's and then with 2  $\mu$ M all-*trans* retinoic acid (ATRA) or vehicle. 24h after ATRA treatment, mRNA levels of (A) CRX, (B) OTX2, and (C) RAR $\beta$  were determined using RT-qPCR. \*p < 0.05 relative to DMSO, \*\*p < 0.01 relative to DMSO, †p < 0.05 relative to scramble siRNA, ††p < 0.01 relative to scramble siRNA, Student's t-test. Error bars indicate standard deviation.

### 3.10 A model for basal OTX2 overexpression in medulloblastoma

In this work, we have characterized the mechanisms of basal OTX2 overexpression in medulloblastoma. Positive regulatory elements of the *OTX2* gene are differentially accessible to *trans*-acting regulators in various medulloblastomas. Such differential accessibility is mediated in part by promoter DNA methylation, but likely involves other factors, including chromatin remodeling complexes, histone modifying enzymes, and activity of *trans*-acting factors themselves. It has been suggested elsewhere that cooperativity between many weak DNA binding factors gives rise to collectively strong

interactions (Levine and Tjian 2003; Song et al. 2011; Neph et al. 2012; Thurman et al. 2012). Our observations that DNase hypersensitive sites are clustered in medulloblastoma and concurrently associate with OTX2 expression status supports this model for the regulation of *OTX2*. Our reporter assays indicate that many such distal enhancers possess activity regardless of OTX2 status, with the exception of DHS 4. Furthermore, we have demonstrated that OTX2 binds to and is required for DHS 4 enhancer activity. If this binding event is the key determinant of OTX2 transcriptional activity, it is likely that the OTX2 transcription factor must be inherited from the tumor cell of origin, as OTX2 protein is required for its own expression and thus cannot be induced *de novo*. This model is supported by our observations that this key enhancer located at DHS 4 exhibits characteristics of an active enhancer in embryonic mouse brain (which includes forebrain, midbrain, and hindbrain), indicating that some normal cell type of the developing brain utilizes this medulloblastoma enhancer element. In accordance with our model, the cell type expressing OTX2 via this enhancer could serve as the cell of origin for OTX2-expressing medulloblastoma, and thus suppression of this enhancer activity during normal development is critical for preventing aberrant OTX2 expression and suppressing tumorigenesis.

### **3.11 A model for retinoid-induced OTX2 repression in medulloblastoma**

Our studies have also uncovered the retinal transcription factor CRX as the intermediate of retinoid-induced OTX2 suppression. We have known for some time that

retinoids inhibit OTX2 expression through an intermediary – the timecourse of OTX2 suppression, which is slow (>3 hours following treatment, data not shown) is consistent with an indirect effect, the translation inhibitor cycloheximide abolishes OTX2 suppression by retinoids, and the *OTX2* promoter lacks a strong retinoic acid receptor binding site. Based on observations in the perinatal mouse eye (Hsiao et al. 2007; Corbo et al. 2010), a weak DHS site in medulloblastoma can be bound by CRX, a gene known to repress OTX2 in this developmental context. Considering the high concentration of retinoids in the developing eye, we were able to extend this regulatory interaction between CRX and OTX2 to the therapeutic effect of retinoids in medulloblastoma. Specifically, treatment of medulloblastoma with retinoids increase chromatin accessibility of a distal cluster of medulloblastoma DHS sites (DHS 1-4 and DHS "R"). DHS "R" would then act as a poised repressor that dominates the enhancer activities of nearby DHS sites to reduce *OTX2* transcription. Thus, modulating CRX induction alone or in combination with retinoid treatment has potential to supplement current medulloblastoma therapy. As OTX2 repression is required for the growth suppressive effect of retinoids in medulloblastoma [Figure 6 and (Bai et al. 2010)], presumably CRX induction is similarly required. Whether CRX expression is sufficient to suppress medulloblastoma tumor growth is yet to be determined and should be investigated in future studies.

## **4. Characterizing the functional consequences of Otx2 overexpression in the postnatal hindbrain**

### ***4.1 Introduction: Animal modeling of medulloblastoma subtypes reveals cell type-specific effects of genetic alterations found in distinct tumor subgroups***

Animal models of medulloblastoma are highly-sought after tools for the purpose of characterizing tumor pathogenesis and as a platform for the preclinical testing of therapeutics. The observation that *Patched*<sup>+/±</sup> mice develop medulloblastoma at low penetrance late in life (Goodrich et al. 1997) initiated studies of the role of the Shh pathway in the pathogenesis of medulloblastoma. Subsequent work has resulted in myriad advances in the field, from the identification of the tumor cell of origin for Shh-driven medulloblastoma (Schuller et al. 2008; Yang et al. 2008), the characterization of cancer stem cell populations in this tumor (Read et al. 2009), and finally the testing of candidate therapeutic strategies in the preclinical setting (Romer et al. 2004). Indeed, the clinical application of Shh pathway inhibitors (Rudin et al. 2009) is a testament to the benefit of animal modeling to designing and testing targeted therapeutics

The molecular characterization of patient medulloblastomas has revealed that the majority of these tumors are not derived from Shh pathway activation (Northcott et al. 2011). Such studies indicate that non-Shh subgroups, particular the Group 3 and 4 tumors, are likely to remain a clinical challenge until targeted therapeutics for these tumor subtypes are also developed.

Animal modeling of non-Shh medulloblastomas is an emerging field, as the majority of animal models of this tumor have utilized activation of the Shh pathway directly (Goodrich et al. 1997; Hallahan et al. 2004; Schuller et al. 2008; Yang et al. 2008) or resulted in Shh pathway activation secondary to tumor suppressor loss (Shakhova et al. 2006; Zindy et al. 2007; Frappart et al. 2009). However, validated genetic models of Wnt subgroup tumors (Gibson et al. 2010) and transplantation models of Group 3 tumors (Kawauchi et al. 2012; Pei et al. 2012) have recently been developed. Specifically, activation of the Wnt pathway in concert with *P53* deletion gives rise to tumors resembling the Wnt molecular subgroup (Gibson et al. 2010) derived from neuronal progenitors of the dorsal brainstem. Similarly, transduction of postnatal cerebellar stem cells or GNPs with degradation-resistant MYC and dominant negative P53 gives rise to Group 3 tumors when transplanted into cerebella of immunodeficient mice (Pei et al. 2012). It is unclear whether these Group 3 mouse tumors are derived from GNPs, postnatal stem cells, or the cells that co-purify with these populations; this ongoing question is perhaps reflective of the challenges of using transplantation models. Still, these and other animal models emphasize that genetic alterations of distinct medulloblastoma subgroups affect specific cell types of the developing hindbrain, despite being broadly activated in some cases (Oliver et al. 2005; Gibson et al. 2010). There is currently no validated model of Group 4 tumors (Eberhart 2012), though a MYCN-driven model has been suggested to share some characteristics of Group 4

patient tumors (Swartling et al. 2010; Swartling et al. 2012). Clearly, genetic models of Group 3 and 4 tumors would be beneficial to understanding the pathogenesis of these tumors in terms of identifying their cellular origins and the functional consequences of particular genetic alterations.

Our studies have focused on designing animal models of Group 3 and 4 tumors by modeling subgroup-specific genetic alterations in the developing hindbrain [(Wortham et al. 2012), to which this chapter refers]. Despite aggressive pursuits to define the mutations present in medulloblastoma, some of the most common focal genetic alterations identified in Group 3 and 4 tumors are copy number gains of *MYC*, *MYCN*, and *OTX2* (Adamson et al. 2010; Jones et al. 2012; Northcott et al. 2012; Pugh et al. 2012; Robinson et al. 2012). These genes may constitute a shared pathway as indicated by the mutually exclusive nature of these alterations (Northcott et al. 2012; Robinson et al. 2012) and the known regulatory interaction between *OTX2* and *MYC* (Adamson et al. 2010). More recent findings have been that tetraploidy is common in these tumors (Jones et al. 2012), as are alterations in chromatin modifier genes such as *CHD7*, *EZH2*, *MLL2/3*, and *UTX* (Parsons et al. 2011; Jones et al. 2012; Pugh et al. 2012; Robinson et al. 2012). Considering that *OTX2* copy number gain is a frequent alteration specifically enriched in Group 3 and 4 medulloblastomas (Adamson et al. 2010), we have sought to derive an animal model of medulloblastoma driven by overexpression of *OTX2* (mouse homolog designated as *Otx2*). Although the role of *OTX2* in tumor maintenance is well-

established (Di et al. 2005; Adamson et al. 2010), the frequency at which it is overexpressed among tumors as well as its broad expression pattern within tumors suggests it may be acquired at early stages of tumorigenesis. Additionally, considering that ectopic expression of OTX2 in different artificial systems have yielded conflicting results (Adamson et al. 2010; Bunt et al. 2010), the importance of assessing OTX2 function in the appropriate tissue context is critical to determine its potential contributions to tumorigenesis.

## **4.2 Methods**

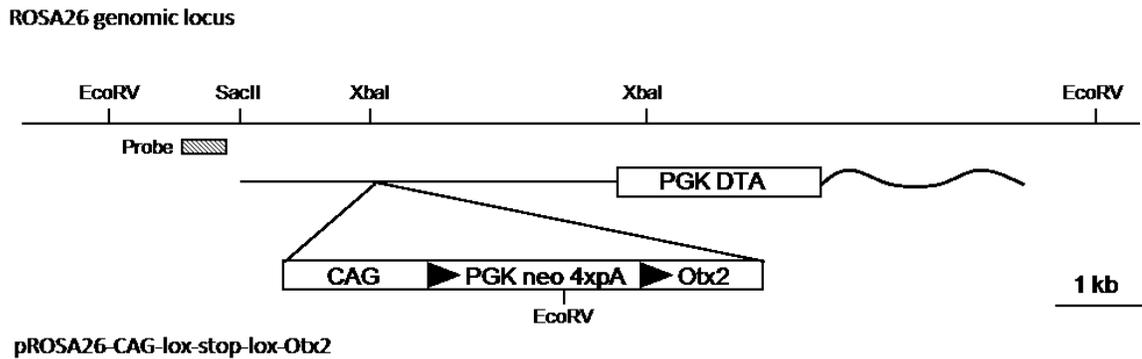
### **4.2.1 Generation of a cre-inducible system for ectopic Otx2 expression *in vivo***

In order to generate an inducible model of Otx2 overexpression in the developing hindbrain, we designed a robust and flexible system that takes advantage of the transgenic mice available for inducing recombination in various cell populations of the CNS. To this end, we generated a knock-in mouse strain (Figure 26) that harbors a cre-inducible Otx2 expression construct inserted into the *ROSA26* locus (Soriano 1999) to ensure active transcription of the insert. The *ROSA26*-targeted construct (Figure 26) was generated by first inserting cDNA for wild type mouse Otx2 into the cloning site of the pBigT construct containing a LoxP-stop-LoxP element to inhibit transcription in the absence of cre-mediated recombination (Srinivas et al. 2001). The resulting construct was then inserted into PacI/AscI sites of the *ROSA26* acceptor plasmid. To maximize expression from this construct, a strong, ubiquitous promoter from the Chicken Actin

Gene (CAG) was inserted into the *PacI* site upstream of the LoxP-stop-LoxP element.

The intervening sequence between the LoxP sites also contains the neomycin resistance gene, which was used for selection of transfected embryonic stem (ES) cells.

The pROSA26-CAG-LoxP-stop-LoxP-Otx2 targeting vector (Figure 26) was linearized with *SfiI* (which was added to the original plasmid with a linker) and then electroporated into 129 Sv/Ev mouse ES cells, and colonies were then selected with G418 to enrich for resistant clones. Following selection, colonies were picked and divided, with one half of each colony replated into a 96-well plate (for maintenance), and the other half lysed for DNA purification and subsequent PCR screening. Colony PCR screening yielded 18 positives of 384 total clones. Twelve clones were then expanded for subsequent Southern blotting to verify proper integration (Figure 27). Finally, of Southern-validated clones, an NLS (nuclear localization signal)-cre expression plasmid was electroporated into expanded cultures, and Otx2 mRNA level was measured by qPCR in both cre-transfected and vector-transfected cultures of each clone (Figure 27). Notably, we observed that (1) the Otx2 inducible construct exhibits minimal leakiness, which is important for the avoidance of off-target phenotypes, and (2) Otx2 inducibility is indeed functional in these knock-in ES cell clones. Both 3A3 and 3B9 clones were then introduced into inner cell masses of C57Bl6 embryos, and the resulting chimeras were then bred with C57Bl6 animals to obtain two stable knock-in strains. The following studies were carried out on the strain derived from the 3A3 clone.



**Figure 26: Targeting strategy and composition of the inducible Otx2 expression construct**

CAG: chicken actin gene, PGK: phosphoglucokinase promoter, neo: neomycin resistance gene, 4xpA: transcriptional stop signal (4x in tandem). "Probe" indicates location of probe for Southern blot. Arrows indicate LoxP elements.

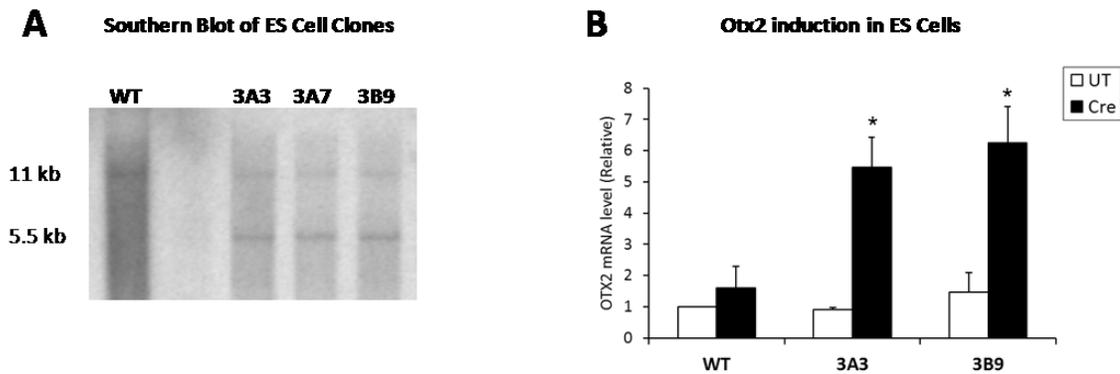
#### 4.2.2 Animal breeding and maintenance

Animals were of mixed strain composition including 129 Sv/Ev, C57Bl6/J, FVB/N, and DBA/2, and animals were genotyped by PCR amplification of DNA purified from tail snips. Animals were monitored regularly for symptoms of brain tumors including head doming, head tilt, ataxia, hunched posture, and lethargy.

#### 4.2.3 Tissue processing and histology

To preserve tissue cytoarchitecture, postnatal days 7-21 (P7-21) and adult mouse brains were fixed *in situ* via intracardiac perfusion first with PBS and then with neutral buffered formalin (NBF). Brains were then removed and post-fixed overnight in NBF. For histological examination, brains were processed for paraffin embedding using standard procedures. To identify focal lesions, brains were sectioned at 5  $\mu$ m thickness

every 300  $\mu\text{m}$  in the sagittal plane, and sections were then hematoxylin & eosin (H & E) stained and examined. Ectopia were defined as basophilic cell clusters not present in any sections of wild type mice. For immunohistochemistry, brains were cryoprotected in 20% sucrose and then embedded in Optimal Cutting Temperature (OCT) medium and frozen at  $-80\text{ }^{\circ}\text{C}$ . 14  $\mu\text{m}$  sections were cut using a cryostat and then stained with the indicated antigens using standard procedures. Fluorescent immunohistochemistry signal was detected with a Nikon Eclipse TE2000-E inverted microscope. The following antibodies were used: goat anti-Otx2 (5 mg/mL) and Phycoerythrin-labelled mouse anti-O4 (1:5) from R & D Systems, mouse anti-Ki67 (1:100) from BD, mouse anti-NeuN (1:100 on paraffin-embedded sections) and rabbit anti-Blbp (1:500) from Millipore, rabbit anti-Pax6 (1:300) from Covance, mouse anti-S100 $\beta$  (1:1000) from Sigma, rabbit anti-Pax2 (1:500) from Invitrogen, rabbit anti-Zic1 (1:400) from Rockland, and rabbit anti-cleaved Caspase 3 (1:100) from Abcam. Specificity of antibody staining was confirmed by staining with non-enriched immunoglobulins of the same class as the experimental antibody.



**Figure 27: Validation of target construct integration and cre inducibility in ES cells**

(A) ES cell clones positive for PCR screening were expanded and harvested for Southern blotting. EcoRV-digested DNA was probed (see Fig 26 for probe location) for the presence of a smaller band, indicative of the additional cut site having been inserted. (B) *Otx2* induction by cre recombinase in ES cells. Southern blot-validated clones were expanded and then electroporated with a cre expression plasmid, and then *Otx2* mRNA level was assessed using RT-qPCR. \* $p < 0.05$  relative to untreated ES cells.

### 4.3 Validation of ROSA26<sup>Lsl-Otx2</sup> mice

To broadly express *Otx2* in the developing mouse brain, we activated the *Otx2* expression construct with the *hGFAP-cre* transgene, which has been shown to express cre recombinase in neural stem cells throughout the embryonic brain (Zhuo et al. 2001; Yang et al. 2008), and should result in inheritance of an activated *Otx2* expression construct in various cerebellar cell types with the exception of Purkinje cells (Yang et al. 2008).

Considering that the cell of origin for *Otx2*-expressing medulloblastomas is not known, expressing *Otx2* in a variety of lineages allows for the simultaneous assessment of the

consequences of ectopic Otx2 expression in various cell types. To maximize expression, we studied mice homozygous for the Otx2 expression construct; thus animals of the genotype *ROSA26<sup>Lsl-Otx2/Lsl-Otx2</sup>, hGFAP-cre* will be referred to as GFAP:Hi-Otx2 animals, whereas *ROSA26<sup>Lsl-Otx2/Lsl-Otx2</sup>* mice (e.g. lacking cre) will serve as the “wild type” control and be referred to accordingly in the text.

Otx2 induction was validated at both the mRNA and protein levels using qPCR and Western blotting of adult cerebellum lysates, revealing that GFAP:Hi-Otx2 mice exhibited ~10-fold induction of Otx2 mRNA relative to wild type (*hGFAP-cre* negative) cerebella (Figure 28). Additionally, the known Otx2-repressed genes ID1 (Bunt et al. 2010) and BDNF (Bunt et al. 2011a) were downregulated in P7 cerebella of GFAP:Hi-Otx2 mice (data not shown), validating that functional Otx2 protein was expressed in this model. Similarly, immunohistochemistry for Otx2 protein revealed that GFAP:Hi-Otx2 mice exhibited an extended Otx2 expression domain (Figure 29A-B), which included intermediate and anterior cerebellar lobes as well as white matter domains of the cerebellum and brainstem. Finally, ectopic Otx2 expression in various hindbrain cell types was confirmed with co-immunofluorescence for Otx2 protein and various cell lineage markers (Figure 29C-O), revealing that Otx2 was induced in GNPs, GABAergic neuronal progenitors, differentiated neurons, oligodendrocytes, astrocytes, and Bergmann glia.

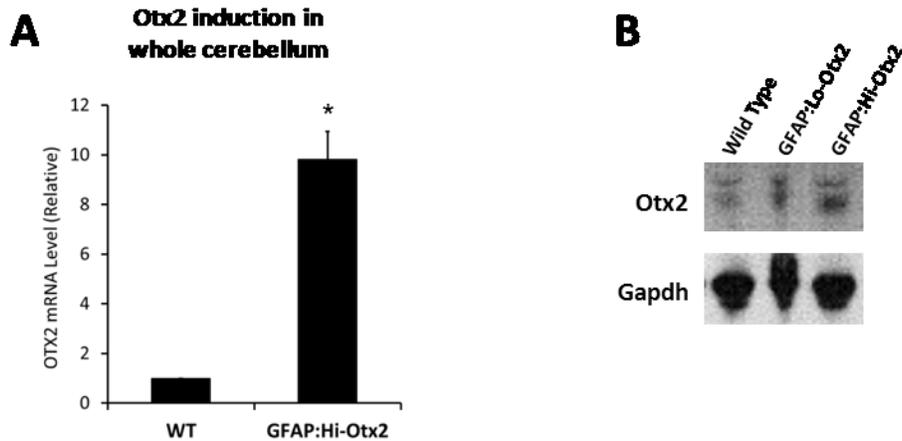


Figure 28: Validation of Otx2 expression in cerebella of GFAP:Hi-Otx2 mice

(A) Whole cerebella from adult GFAP:Hi-Otx2 mice or wild type (*cre*<sup>-</sup>) controls were harvested, and Otx2 mRNA level was measured with RT-qPCR. (B) Otx2 protein level was determined in whole adult cerebella of wild type (*Lsl-OTX2* only), GFAP:Lo-Otx2, and GFAP:Hi-Otx2 mice.

## 4.4 Characterizing the effects of Otx2 upon animal survival and hindbrain development

### 4.4.1 Effect of ectopic Otx2 expression upon survival

To determine the ability of Otx2 to initiate medulloblastoma, we generated a cohort of GFAP:Hi-Otx2 mice (n=9) and monitored them for up to one year for the development of overt symptoms of brain tumors. GFAP:Hi-Otx2 animals exhibited reduced postnatal growth (not shown), and females were hypofertile. We observed lethargic and moribund GFAP:Hi-Otx2 animals at various postnatal and adult timepoints (Figure 30). However, when these brains were serially sectioned and H & E stained, none of these animals exhibited histologically-apparent brain tumors. Additionally, brains from one year old animals appeared grossly normal (Figure 31A).

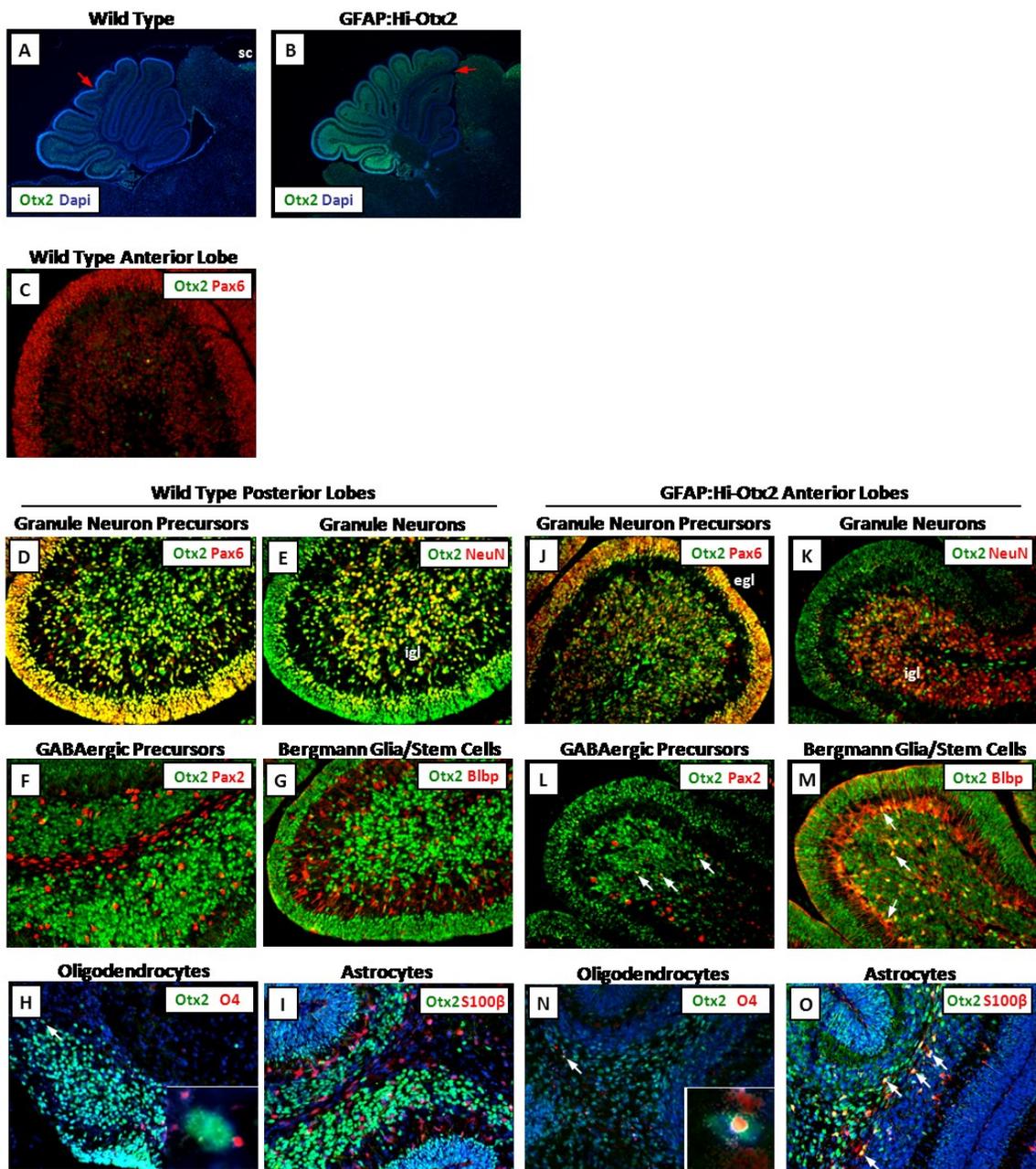
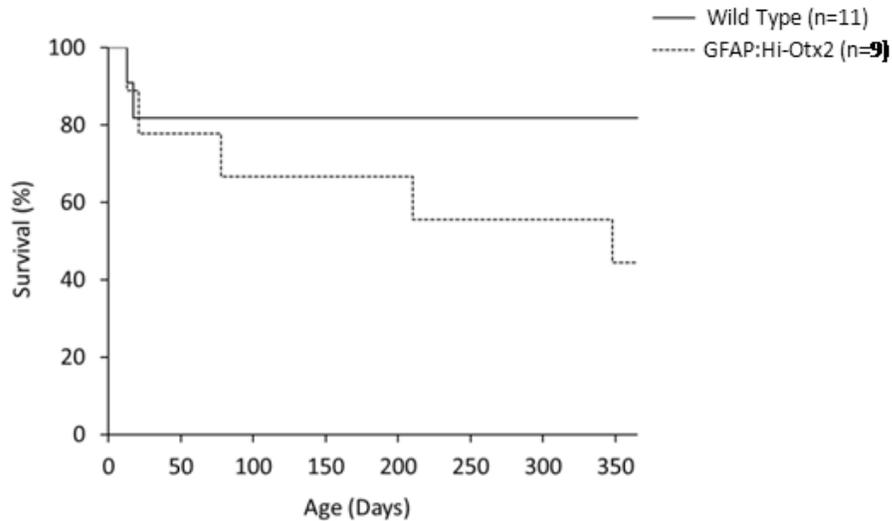


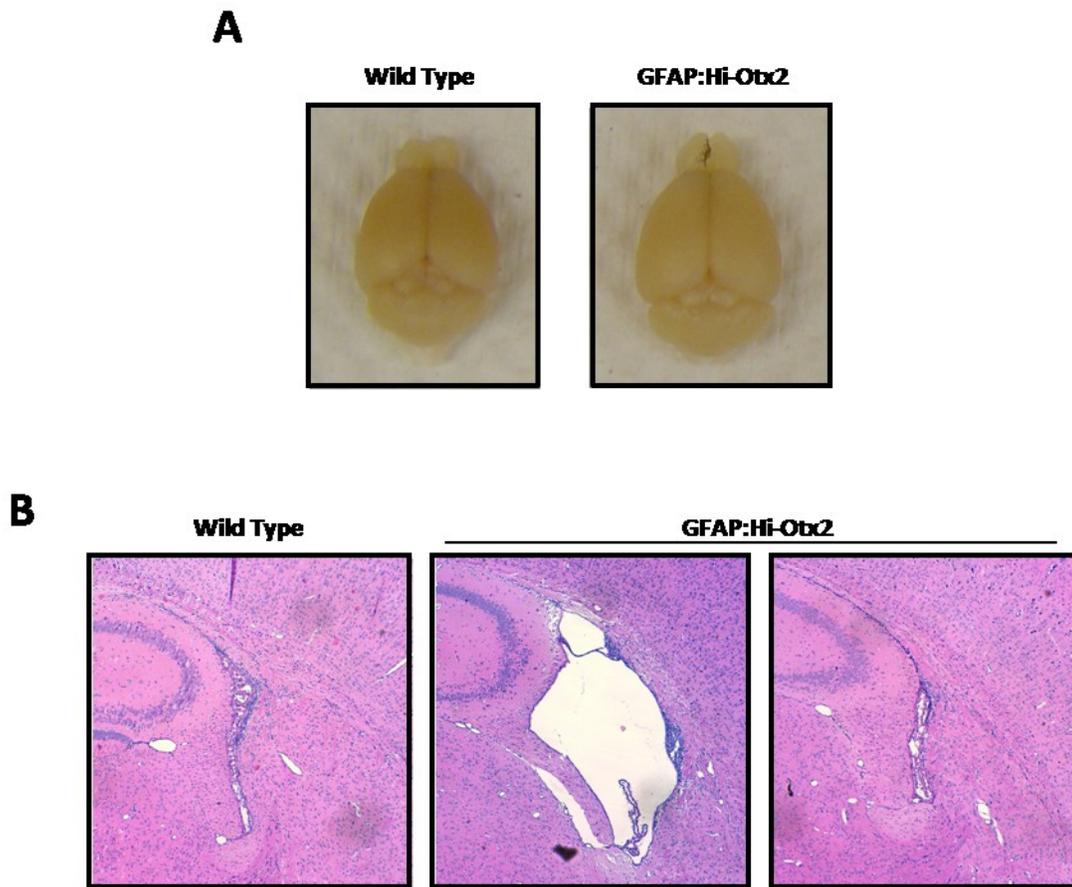
Figure 29: Validation of Otx2 expression in diverse cell types of the cerebellum  
 (A-B) Low-power (4x) images of (A) wild type and (B) GFAP:Hi-Otx2 P7 cerebella stained for Otx2 with immunohistochemistry. (C-O) P7 cerebella stained with the indicated antibodies demonstrating co-staining of various cell types of the anterior cerebellum in GFAP:Hi-Otx2 mice. Insets: cropped and enlarged images from arrows. Images shown at 20x magnification.



**Figure 30: Survival of GFAP:Hi-Otx2 mice**

**Animals were monitored regularly for symptoms of brain tumors, and sacrificed when exhibiting overt morbidity.**

To further investigate the potential cause of death induced by ectopic Otx2 expression in the CNS, we serially sectioned brains of P21 mice coronally through the entire length of the brain to identify defects in the ventricular system that may contribute to morbidity. Although we did not observe obstructions of the ventricular system (e.g. at the aqueduct), we did confirm expansion of the lateral ventricles by at least 50% in 3/4 animals (Figure 31B), indicative of mild hydrocephalus. Ventricular expansion was also observed in lethargic GFAP:Hi-Otx2 animals (data not shown). Although it is unclear whether ectopic Otx2 expression induced some undetected obstruction or rather affected cerebrospinal fluid production, circulation, or composition, the ventricular swelling observed in otherwise healthy mice may over the long term contribute to some of the morbidities observed in these animals (Figure 30).



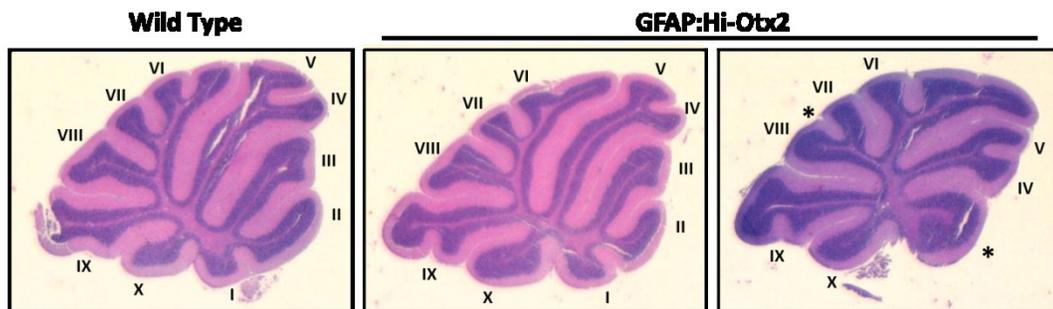
**Figure 31: Brain morphology of GFAP:Hi-Otx2 mice**

**(A) One year old GFAP:Hi-Otx2 mouse brains appear grossly normal. (B) H & E stained coronal sections of postnatal day 21 GFAP:Hi-Otx2 mice reveals mild hydrocephalus in some animals.**

#### **4.4.2 Effect of ectopic Otx2 expression upon hindbrain development**

Having determined that Otx2 overexpression is not sufficient to give rise to medulloblastoma, we then sought to determine the course of hindbrain development in GFAP:Hi-Otx2 mice. As the cerebellum proliferates massively from P0 to P21, we

focused upon this stage of hindbrain development. Overtly, in midline H & E stained sections, we observed variable foliation defects of anterior cerebellar lobes in GFAP:Hi-Otx2 mice (Figure 32), whereas some animals exhibited truncated lobes II and III, others exhibited a single spherical (rather than laminar) lobe in place of lobes I-III, while a subset of animals exhibited proper cerebellar foliation (Figure 32).

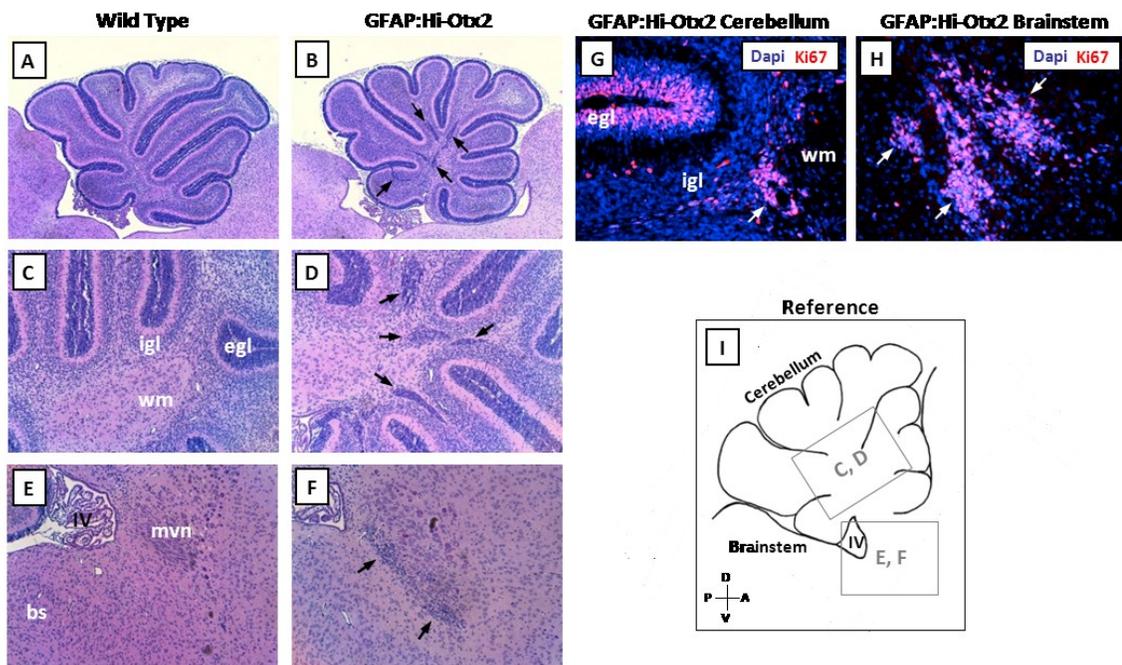


**Figure 32: Cerebellar morphology of GFAP:Hi-Otx2 mice**

**Representative H & E-stained cerebellar sections from P21 mice are shown. Folia are labeled. Note foliation defects (starred) are variable in GFAP:Hi-Otx2 mice, with some animals exhibiting normal foliation.**

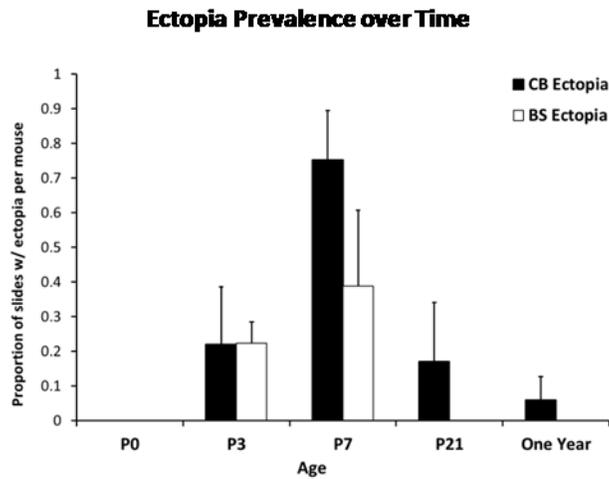
More focally, examination of serially-sectioned postnatal brains revealed the accumulation of ectopic cell clusters in the white matter of the cerebellum and brainstem at P3 and P7. Histologically, these ectopia resembled deeply basophilic, densely-packed clusters of cells exhibiting granular nuclei (Figure 33), reminiscent of both neuronal progenitor cells as well as preneoplastic lesions identified in various other animal models that eventually develop medulloblastoma (Oliver et al. 2005; Gibson et al. 2010).

The ectopia were very prevalent in the cerebellum, being identified in nearly every single section ( $75\% \pm 14\%$ , Figure 34) in every mouse at P3 and P7 ( $n=8$ ), and in the brainstem, the ectopia were identified in most animals (present in 7/8 animals at P3 or P7) but were found at more discrete locations (Figure 34 and data not shown). Notably, these ectopia were not histologically detectable at P0, and were quite rare and more diffusely-clustered in P21 and adult animals, indicating that they were generated postnatally and resolved as animals reached adulthood (Figure 34).



**Figure 33: Focal hyperplasia in GFAP:Hi-Otx2 mice**

(A-F) H & E stained sections of P7 hindbrains from wild type (A, C, E) or GFAP:Hi-Otx2 (B, D, F) mice. (G-H) Immunohistochemistry for Ki67 in the cerebellum (G) and brainstem (H) of P7 GFAP:Hi-Otx2 mice. (I) Cartoon illustrating the location of field depicted in (C-F). Arrows indicate ectopia. Fields A-B are shown at 4x magnification, fields C-F are shown at 10x magnification, and fields G-H are shown at 20x magnification.



**Figure 34: Prevalence of ectopia in GFAP:Hi-Otx2 mice over time**

**H & E stained sections were examined for the presence of ectopic cell clusters, defined as basophilic, granular cells not present in any wild type animal, at various timepoints. CB: cerebellum, BS: brainstem.**

Immunohistochemistry for the proliferation marker Ki67 revealed that ectopic cells of both domains were predominately engaged in the cell cycle at P7 (Figure 33). Considering that these ectopia are resolved over time, these cells presumably exit the cell cycle or undergo cell death at some point. To determine the fate of ectopia, we immunostained P7 and P21 brain sections with markers of apoptosis (cleaved Caspase 3) and differentiation (Zic1 and NeuN, both neuronal differentiation markers). While there was scant apoptosis in these cell clusters (Figure 35), we did observe staining with the early differentiation marker Zic1 at the periphery of ectopia at P7 and more uniform induction of the late differentiation marker NeuN in the diffuse cerebellar ectopia of P21 mice (Figure 35). Notably, we did not observe histological indicators of necrosis (such as

pyknotic nuclei) at any of these timepoints. Collectively, these results suggest that ectopia are first generated postnatally, proliferate transiently, and then differentiate and disperse into the white matter to eventually become histologically indistinguishable from normal cell populations in these domains.

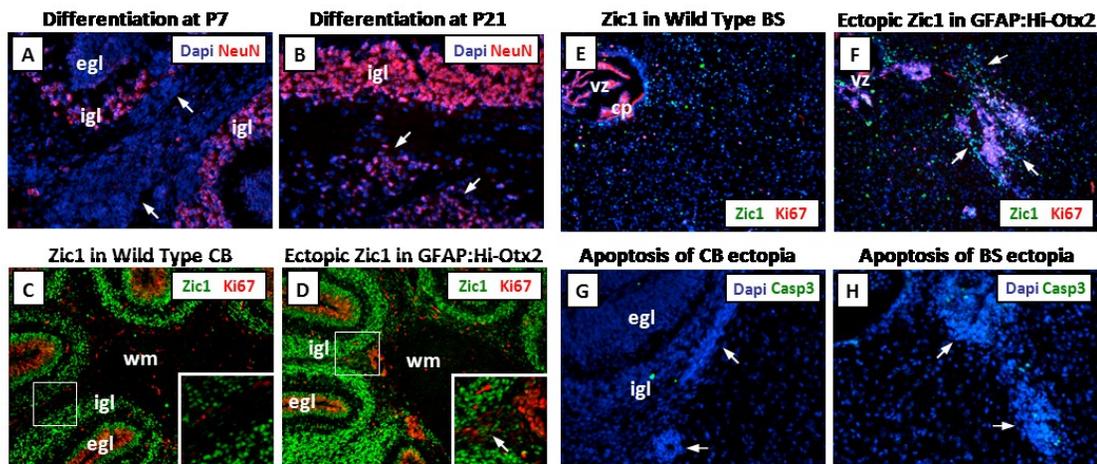


Figure 35: Fate of ectopia in GFAP:Hi-Otx2 mice

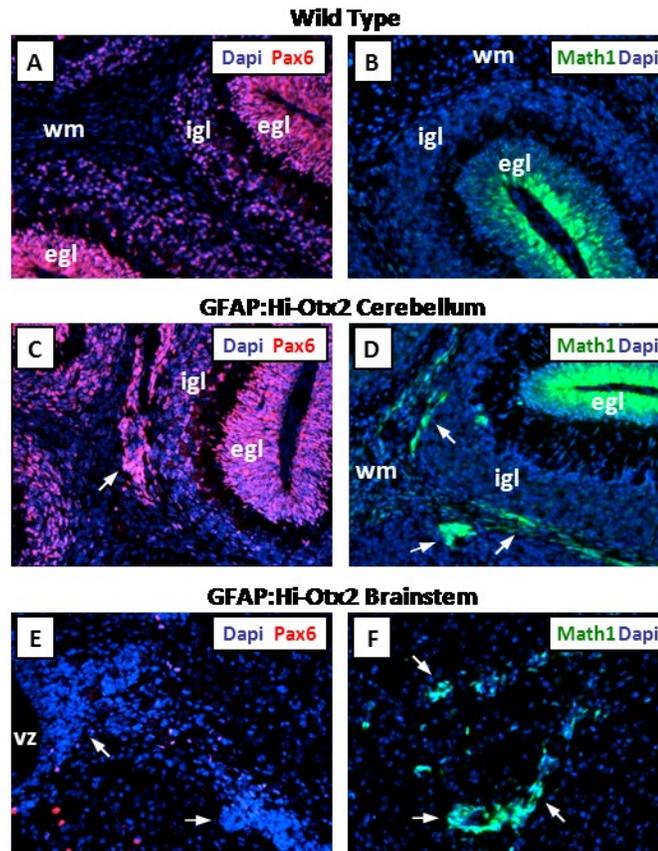
P7 (A, C-H) or P21 (B) cerebella from wild type (C, E) or GFAP:Hi-Otx2 mice were stained with the indicated antibodies. Arrows indicate ectopia. All images shown at 20x magnification. CB: cerebellum. BS: brainstem.

## 4.5 Investigation of the distinct effects of *Otx2* upon neuronal progenitor cells

### 4.5.1 Identification of neuronal progenitor cells as being distinctly responsive to *Otx2* overexpression

By broadly modeling the genetic alterations found in distinct medulloblastoma subgroups, it has been possible to identify cell types that distinctly respond to such alterations, which often correspond to the cellular origins of that particular tumor

(Oliver et al. 2005; Gibson et al. 2010). Thus, characterization of the cell types comprising ectopically proliferating cells would reveal which cell lineages are distinctly affected by ectopic Otx2 expression. Immunohistochemistry for various cell lineage markers revealed that hyperplasias were predominately comprised of neuronal progenitor cells (Figure 36 & 37). Furthermore, examination of these slides revealed normal morphology and localization of other hindbrain cell types in postnatal GFAP:Hi-Otx2 mice (Figure 37 and data not shown). These results suggest that Otx2 overexpression distinctly affects neuronal progenitor cells, and that this effect is not clearly associated with altered migratory scaffolds provided by supporting glia. While ectopia from both cerebellum and brainstem exhibited characteristics of neuronal progenitor cells [(namely expression of the Math1-GFP reporter allele (Figure 36) and association with ectopic Zic1<sup>+</sup> cells (Figure 35 and 37)], the molecular phenotype of ectopia from the two domains are distinct, such that cerebellar ectopia express the granule neuron lineage marker Pax6, which was not expressed by brainstem ectopia (Figure 36). While the particular brainstem neuronal progenitor cell comprising these ectopia is currently unknown, an investigation of more specific neuronal markers and the induction of Otx2 in increasingly-specific domains of the precerebellar system should clarify their spatial origins and lineage identity.



**Figure 36: Ectopia in GFAP:Hi-Otx2 mice resemble neuronal progenitor cells**

Hindbrains from P7 (A, B) wild type or (C-F) GFAP:Hi-Otx2 mice were stained with the indicated antibodies (A, C, E) or assessed for GFP fluorescence from the *Math1-GFP* reporter allele (B, D, F). Arrows indicate ectopia. All images shown at 20x magnification.

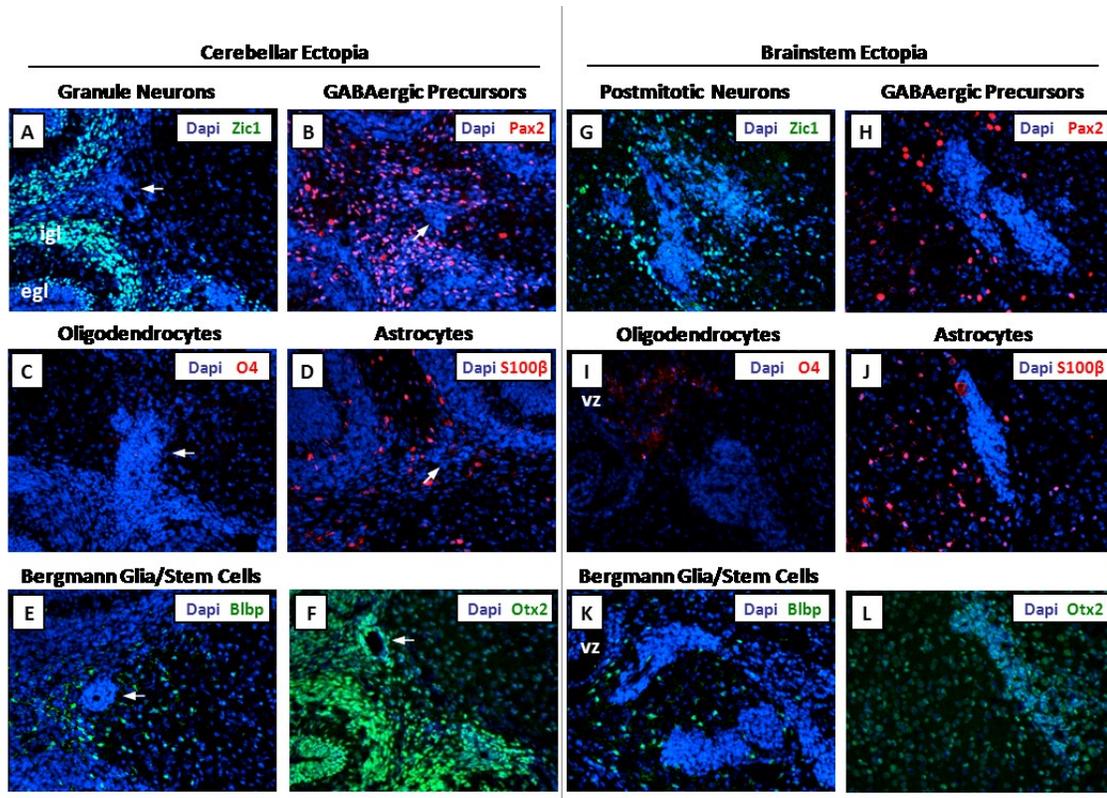


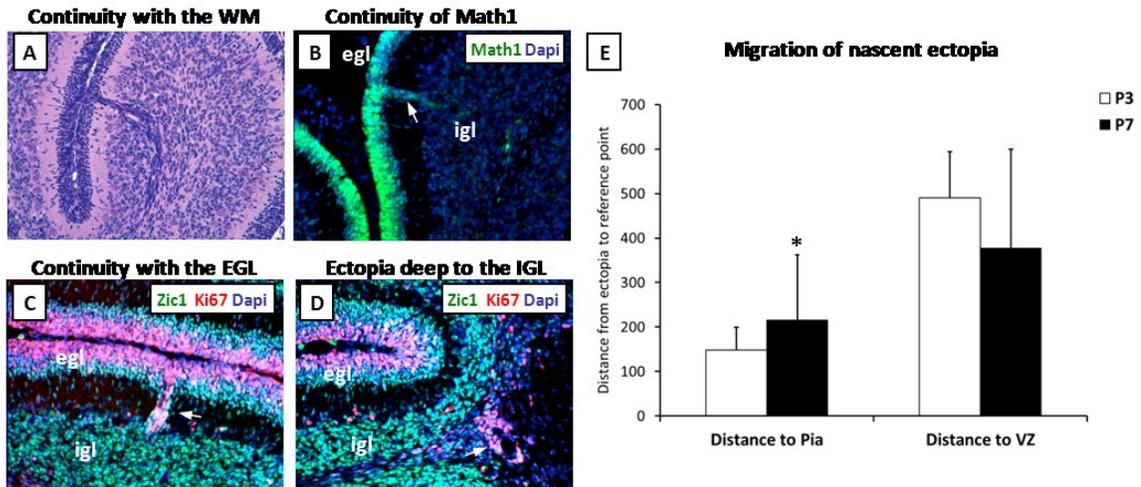
Figure 37: Staining for cell lineage markers and Otx2 protein in ectopia

P7 cerebella (A-F) or brainstems (G-L) were stained with the indicated antibodies and shown at 20x magnification. Arrows indicate ectopia.

#### 4.5.2 Validation of a migratory defect in cerebellar ectopia

The appropriate spatiotemporal regulation of GNP proliferation is critical to proper cerebellar development and suppression of medulloblastoma formation (Oliver et al. 2005; Dey et al. 2012). Considering that these cells are a known cell of origin for medulloblastoma and their coordinated spatiotemporal proliferation and differentiation processes are well-characterized (Wechsler-Reya and Scott 1999; Choi et al. 2005), we determined the spatial origin of these ectopic GNPs in GFAP:Hi-Otx2 mice. First, we

noted rare cellular raphes contiguous with the EGL that exhibited features of proliferating GNPs (expression of Ki67 and the Math1-GFP reporter and lack of Zic1 expression), much like the ectopia found in the white matter (Figure 38). To determine if these ectopia were derived from the EGL and destined for the white matter, we determined their localization as they were being actively generated. To this end, we measured the distance of ectopia relative to either the cerebellar pial surface or to the roof of the IVth ventricle in cerebellar sections of P3 and P7 animals (Figure 38E). These spatial indicators suggested that ectopia were indeed being produced near the cerebellar surface and migrating deep into the white matter. This finding suggests that Otx2 expression permits GNP migration from the EGL past the internal granule layer (IGL) and deep into the cerebellum. This effect is consistent with a role for Otx2 in permitting mitogenic niche-independent proliferation, resistance to differentiation signals in the IGL, and disruption of migrational guidance. These findings are reflective of Otx2 affecting hallmark cancer pathways (Hanahan and Weinberg 2000) including growth factor-independent proliferation, resistance to cell cycle checkpoints, and migration (perhaps related to tumor metastasis) in untransformed hindbrain cells.



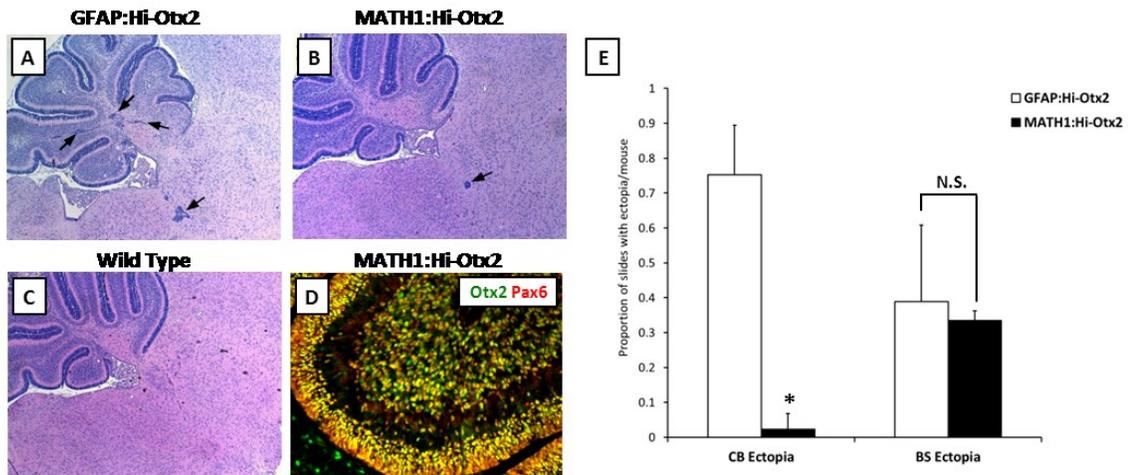
**Figure 38: Spatial origin of ectopia**

(A-D) P7 cerebella from GFAP:Hi-Otx2 mice were H & E stained (A) or stained with the indicated antibodies (B-D). (E) P3 and P7 cerebella were H & E stained and then the location of ectopia relative to the pial surface or roof of the fourth ventricle (VZ) was measured. Distance is presented as microns.

### 4.5.3 Partial phenocopy of the focal hyperplasia phenotype in *Math1*:Hi-Otx2 mice

We then sought to determine if Otx2 can give rise to ectopic proliferation in the hindbrain when its expression is limited to neuronal progenitor cells. To this end, we generated animals in which the Otx2 expression construct was activated specifically in rhombic-lip derived neuronal progenitor cells by inducing the *ROSA26<sup>Lsl-Otx2</sup>* construct with the *Math1-cre* transgene (Matei et al. 2005). These animals give rise to recombinant GNPs (Yang et al. 2008) and neuronal progenitor cells of the precerebellar system (Grammel et al. 2012), which we validated to express Otx2 in our model using co-immunofluorescence for Otx2 and neuronal progenitor cell markers (Figure 39). Examination of H & E-stained sections from these animals revealed that ectopia of the

brainstem, but not the cerebellum, were recapitulated using this system of Otx2 induction (Figure 39E). These results indicate that Otx2 can cell-autonomously give rise to ectopic proliferation of neuronal progenitor cells of the precerebellar system.



**Figure 39: Restricting Otx2 expression to Math1-expressing neuronal progenitor cells partially phenocopies *hGFAP-cre* induction of Otx2**

(A-C, E) H & E stained sections were examined for the presence of ectopic cell clusters. (D) P7 cerebella from *Math1:Hi-Otx2* mice were stained with the indicated antibodies. CB: cerebellum, BS: brainstem.

The mechanisms whereby Otx2 gives rise to cerebellar ectopia in *GFAP:Hi-Otx2* mice but not *Math1:Hi-Otx2* mice are currently unclear. Possibilities remain that (1) expression of Otx2 in neural stem cells, but not GNPs, cell-autonomously gives rise to GNPs that exhibit these abnormal proliferative and migratory phenotypes (e.g. the

neural stem cell to GNP differentiation process is altered), (2) the effect of Otx2 upon GNPs occurs cell non-autonomously, or (3) ectopic localization is stochastic among GNPs and thus relative to total recombined cells in GFAP:Hi-Otx2 mice, whereas Math1:Hi-Otx2 mice do not recombine a sufficient quantity of GNPs to give rise to detectable ectopia. Our observations that Math1:Hi-Otx2 mice have undergone extensive recombination and robust Otx2 induction in P7 cerebella (Figure 39D and data not shown) leads us to favor either of the first two models.

## ***4.6 Determining the ability of co-occurring genetic alterations to cooperate with Otx2 to form medulloblastoma***

### **4.6.1 Homozygous *P53* deletion does not induce highly-penetrant medulloblastoma in GFAP:Hi-Otx2 mice**

Our studies have thus far established that Otx2 alters migrational guidance and proliferation of hindbrain neuronal progenitor cells; however, the ability of these ectopic cells to give rise to medulloblastoma is unknown. Validation of this potential would prove that these hyperplasias are indeed precursors to medulloblastoma and can serve as cells of origin for Otx2-expressing tumors. *P53* deletion alone using the *hGFAP-cre* transgene has been shown to give rise to medulloblastoma at a low rate (~10%) (Wang et al. 2009). Although *P53* mutations are rare in non-Shh/non-Wnt medulloblastomas, conditional or conventional *P53* null mice serve as a tool to inhibit oncogene-induced failsafe mechanisms (Serrano et al. 1997) and nonspecifically induce additional genetic alterations (Wang et al. 2009). Such an approach has been validated to cooperate with

established medulloblastoma initiation events such as constitutive Shh or Wnt pathway activation (Uziel et al. 2005; Gibson et al. 2010). As such, we have sought to combine *P53* deletion with *Otx2* overexpression in the GFAP:Hi-*Otx2* mice by generating *ROSA26<sup>Lsl-Otx2/Lsl-Otx2</sup>, P53<sup>fl/fl</sup>, hGFAP-cre* animals [using the strain described in (Jonkers et al. 2001)], hereafter referred to as GFAP:Hi-*Otx2*, *P53<sup>fl/fl</sup>* mice, and monitoring them for medulloblastoma formation. This genotype combination would give rise to *Otx2*-expressing hindbrain cells that resist oncogene-induced senescence or apoptosis and harbor substantial genomic instability, which could at an appreciable rate give rise to additional driver events in medulloblastoma formation. Crossing *P53<sup>fl/fl</sup>* alleles into the GFAP:Hi-*Otx2* mice proved to be problematic due to the extremely low fertility of *ROSA26<sup>Lsl-Otx2/Lsl-Otx2</sup>, P53<sup>fl/fl</sup>* females. Regardless, aging of a small cohort (n=5) of these GFAP:Hi-*Otx2*, *P53<sup>fl/fl</sup>* animals for up to one year revealed that this genotype combination does not give rise to medulloblastoma at high penetrance (Table 4 and Figure 40). As described above, GFAP:Hi-*Otx2* animals exhibit some premature death, which was also apparent in GFAP:Hi-*Otx2*, *P53<sup>fl/fl</sup>* animals (Figure 40 and 41). As such, we also generated conditional *P53* null mice heterozygous for the knock-in construct (e.g. *ROSA26<sup>Lsl-Otx2/+</sup>, P53<sup>fl/fl</sup>, hGFAP-cre*) to circumvent this premature lethality. Similar to *P53<sup>fl/fl</sup>, hGFAP-cre* animals [Table 4, Figure 41, and (Wang et al. 2009)], these mice developed tumors on their flanks that histologically resembled sarcomas (data not shown) and exhibited a low incidence of glioma formation (as indicated by GFAP

positivity of intracranial tumors; data not shown), but did not develop medulloblastomas.

**Table 4: Spectrum of tumors and survival for mice harboring various combinations of Otx2 expression and tumor suppressor deletions.**

Animals were monitored regularly for symptoms of brain tumors.  
Data are shown for postweaning animals only.

	GFAP-cre	ROSA26 <sup>lox</sup> -Otx2/Lox-Otx2	GFAP:Lo-Otx2	GFAP:Lo-Otx2, P53 <sup>fl/fl</sup>	GFAP:Hi-Otx2	GFAP:Hi-Otx2, P53 <sup>fl/fl</sup>	GFAP-cre, P53 <sup>fl/fl</sup>	GFAP:Lo-Otx2, P53 <sup>fl/fl</sup> , PTEN <sup>fl/fl</sup>	GFAP:Hi-Otx2, P53 <sup>fl/fl</sup> , PTEN <sup>fl/fl</sup>
# Brain tumors	0	0	0	11.1	0	0	15.4	0	0
# Flank tumors	0	0	0	33.3	0	0	53.8	0	0
# Other causes of death	0	0	27.3	22.2	42.9	40	23.1	20.0	16.7
% Surviving one year	100	100	72.7	33.3	57.1	60	7.7	80.0	83.3
Total # of mice	7	11	11	9	7	5	13	5	6

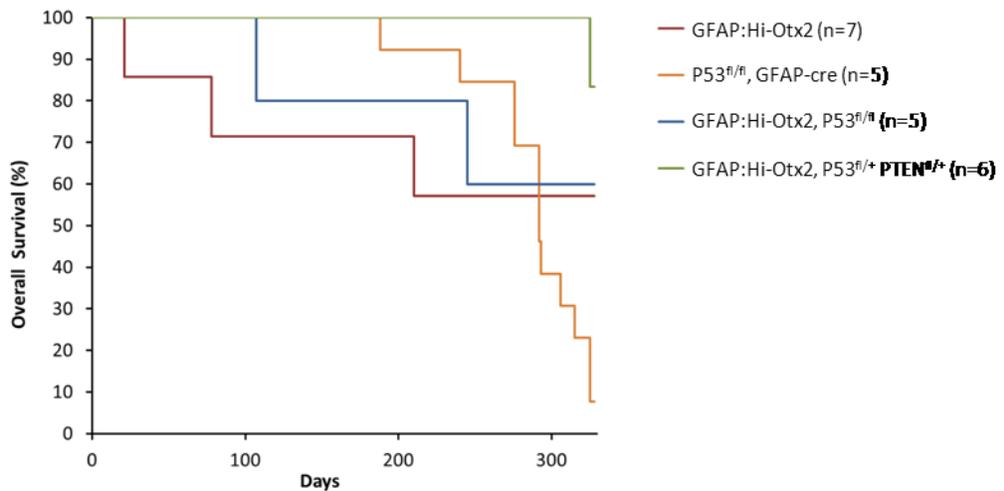


Figure 40 (continued): Overall survival of mice expressing Otx2 and lacking various tumor suppressors

Animals were monitored for the presence of symptoms of brain tumors or other indications of morbidity (such as the presence of large flank tumors) and sacrificed when symptomatic. Data are shown for postweaning animals only.

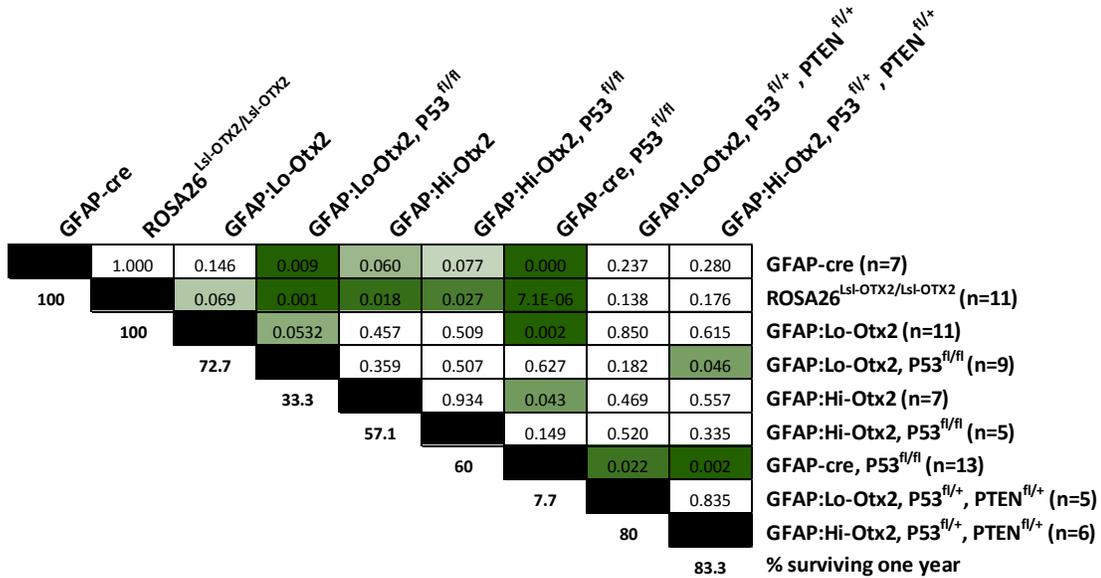


Figure 41: Pairwise survival comparisons between mice expressing Otx2 and lacking various tumor suppressors

Log rank test *p*-values are presented for pairwise comparisons of the indicated genotypes. Cohorts of animals were monitored for up to one year of age to generate survival curves. Percentage of animals of each genotype (indicated at the top of each column) surviving the entire year is indicated at the bottom of each column. Data are shown for postweaning animals only.

#### 4.6.2 Genetic, but not functional, evidence for cooperation of *PTEN* and *P53* heterozygous loss with *OTX2* alterations

A rational approach to combining Otx2 expression with candidate cooperating genetic events would be to model the events that are found to co-occur with *OTX2*

expression or copy number gain in patient tumors. However, the common genetic alterations of Group 3 and 4 medulloblastomas (which distinctly harbor *OTX2* copy number gain) are mostly limited to copy number gains of *CDK6*, *MYC*, *MYCN*, and *OTX2* itself (Figure 42). Notably, these alterations are largely mutually exclusive (Northcott et al. 2009b; Northcott et al. 2012), and considering that *OTX2* can transcriptionally regulate *MYC*, these genes may constitute a single pathway. Additionally, while epigenetic regulators such as *CHD7*, *EZH2*, *MLL2/3*, and *UTX* are altered in Group 3 and 4 tumors, animal models for these loss-of-function and gain-of-function events are unavailable or have only recently been developed. However, co-occurring heterozygous losses of the classic tumor suppressors *PTEN* and *P53*, often part of broad chromosomal events (Northcott et al. 2009b; Northcott et al. 2011), have been described in Group 3 and Group 4 medulloblastoma and do co-occur at a high frequency with *OTX2* copy number gains (Figure 42).

As such, we generated combinations of *ROSA26<sup>Lsl-Otx2/+</sup>*, *P53<sup>fl/+</sup>*, *PTEN<sup>fl/+</sup>*, *hGFAP-cre* (*GFAP:Lo-Otx2*, *P53<sup>fl/+</sup>*, *PTEN<sup>fl/+</sup>*) mice and *ROSA26<sup>Lsl-Otx2/Lsl-Otx2</sup>*, *P53<sup>fl/+</sup>*, *PTEN<sup>fl/+</sup>*, *hGFAP-cre* (*GFAP:Hi-Otx2*, *P53<sup>fl/+</sup>*, *PTEN<sup>fl/+</sup>*) mice [using the *PTEN<sup>fl</sup>* strain described in (Trotman et al. 2003)] to model these genetic alterations found to frequently co-occur in medulloblastoma. However, cohorts of these animals monitored for up to 11 months of age did not develop medulloblastoma or other brain tumors. Thus, the genetic alterations that can promote tumorigenesis in cooperation with aberrant *Otx2* expression

are yet to be determined. Future studies should address the contribution of rare alterations in chromatin modification and remodeling genes in the pathogenesis of Otx2-driven medulloblastoma, as well as the role of Otx2 in tumorigenesis of other animal models that generate Otx2-expressing medulloblastomas, such as the *Gl1-tTA:TRE-MYCN/Luc* (Swartling et al. 2010) or *Ctnnb1<sup>+/fl(ex3)</sup>, p53<sup>fl/fl</sup>, Blbp-cre* mice (Gibson et al. 2010).

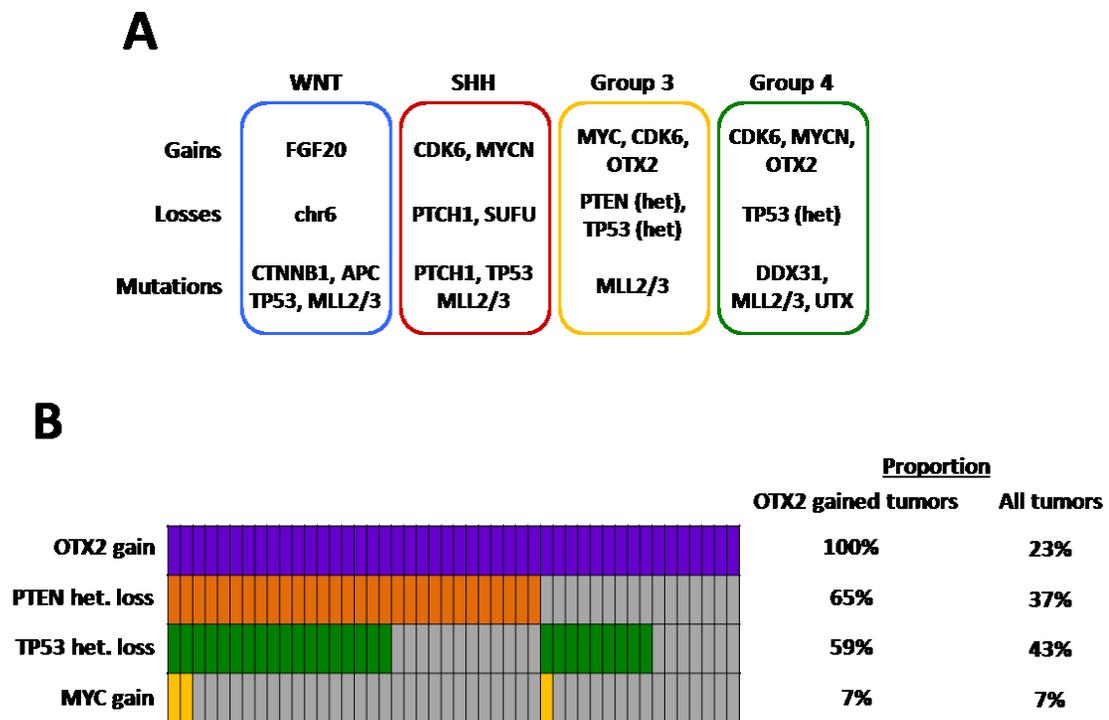


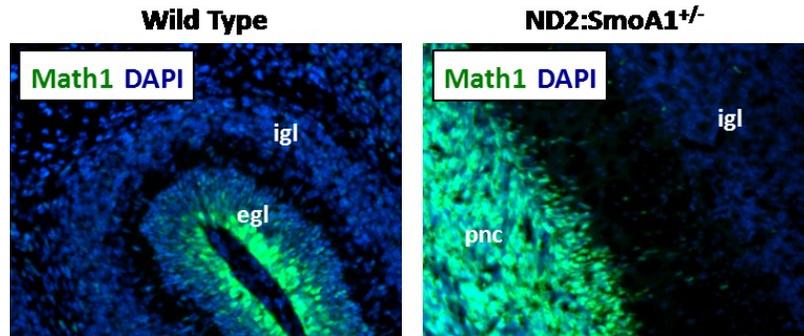
Figure 42: Genetic alterations present in medulloblastoma subtypes and individual tumors harboring *OTX2* copy number gain

(A) Subgroup-specific genetic alterations in medulloblastoma, from (Lindsey et al. 2011; Northcott et al. 2011; Jones et al. 2012; Pugh et al. 2012; Robinson et al. 2012). (B) Copy number alterations enriched in individual *OTX2*-gained tumors, from (Northcott et al. 2009).

### 4.6.3 The effect of Otx2 upon tumor promotion in medulloblastomas initiated by constitutive Shh pathway activation

The animal model discussed above investigated the potential for Otx2 to initiate medulloblastoma tumorigenesis. However, our previous studies in established medulloblastomas indicate that Otx2 plays a role in tumor maintenance and progression. As such, we tested the ability of Otx2 to contribute to medulloblastoma formation in the context of an initiating mutation. To this end, we combined our inducible Otx2 expressing mouse with the *ND2:SmoA1* strain, which expresses a constitutively active mutant of the Shh pathway transducer *Smo* in cerebellar GNPs (Hallahan et al. 2004). *ND2:SmoA1<sup>+/-</sup>* mice are predisposed to the development of Shh subtype medulloblastomas (Hallahan et al. 2004). Although Otx2 expression is excluded from this medulloblastoma subtype in patients, the *ND2:SmoA1<sup>+/-</sup>* strain is a robust model of medulloblastoma initiation, developing these tumors at approximately 50% penetrance by six months of age (Hallahan et al. 2004). Furthermore, the potential for medulloblastomas to interchange among the four subtypes is currently unclear; for example, heterozygous deletion of *PTEN* in *ND2:SmoA1<sup>+/-</sup>* mice suppresses the Shh pathway and gives rise to a nodular histology (Castellino et al. 2010), indicating that the molecular and histological phenotype of medulloblastoma may transition in response to particular genetic alterations. Reminiscent of *Patched<sup>+/-</sup>* mice (Oliver et al. 2005), tumor formation in *ND2:SmoA1<sup>+/-</sup>* mice is preceded by a preneoplastic state in which GNP cells form Math1-expressing rests or masses at the cerebellar surface (Figure 43). As such,

induction of *ROSA26<sup>Lsl-Otx2</sup>* via the *Math1-cre* transgene (Matei et al. 2005) will initiate Otx2 expression at an early stage of tumorigenesis in this model.



**Figure 43: Preneoplastic lesions of ND2:SmoA1 mice express Math1**

*Math1-GFP* transgenic mice on either a wild type or ND2:SmoA1<sup>+/-</sup> background were assessed for GFP fluorescence at either P7 (Wild Type) or adulthood (ND2:SmoA1<sup>+/-</sup>).

ND2:SmoA1<sup>+/-</sup> animals expressing high level of Otx2 (e.g. ND2:SmoA1<sup>+/-</sup>, *ROSA26<sup>Lsl-Otx2/Lsl-Otx2</sup>*, *Math1-cre*, subsequently referred to as SmoA1, Math1:Hi-Otx2 mice) by essence of *Math1-cre* activation exhibited a trend towards decreased tumor latency relative to control ND2:SmoA1<sup>+/-</sup>, *ROSA26<sup>Lsl-Otx2/Lsl-Otx2</sup>* mice; however, this survival difference did not reach significance (Figures 44 and 45;  $p = 0.3726$ , log-rank test) with the current cohort sizes and timepoints. Assessment of larger cohorts of SmoA1, Math1:Hi-Otx2 mice should give more power to this statistical comparison. Histological examination of tumors derived from SmoA1, Math1:Hi-Otx2 mice did not reveal clear morphological differences between these and control tumors; namely, tumors from both genotypes exhibited moderate anaplasia as indicated by apoptotic cells distributed throughout the tumor (Figure 46) but lack of nuclear enlargement, wrapping, or other

pleomorphism characteristic of severely anaplastic or large cell anaplastic medulloblastoma. The current data support a potential role for Otx2 to contribute to tumorigenesis through effects upon tumor promotion, although additional studies will be necessary to validate the trend towards decreased tumor latency. Although tumor histology was largely unaffected by Otx2, further studies of tumor molecular phenotype (i.e. microarray analysis of tumors or immunohistochemical detection of subtype-specific antigens) will be required to determine if Otx2 actually alters the tumor phenotype or rather accelerates the formation of phenotypically-equivalent medulloblastomas.

It should be noted that control mice homozygous for  $ROSA26^{Lsl-Otx2}$  exhibited decreased tumor latency relative to control animals heterozygous for this knock-in allele (Figures 44-45). This effect may indicate a contribution of the endogenous  $ROSA26$  transcripts or rather a suppressive effect of high *neo* expression from the unrecombined  $ROSA26^{Lsl-Otx2}$  construct to tumorigenesis. We cannot rule out the contribution of the *neo* gene (which is only deleted in *SmoA1*, *Math1:Hi-Otx2* mice) to our observations. Similar effects could also explain the reduced prevalence of flank tumors observed in *GFAP:Hi-Otx2*,  $P53^{fl/fl}$  mice (Figure 41, Table 4).

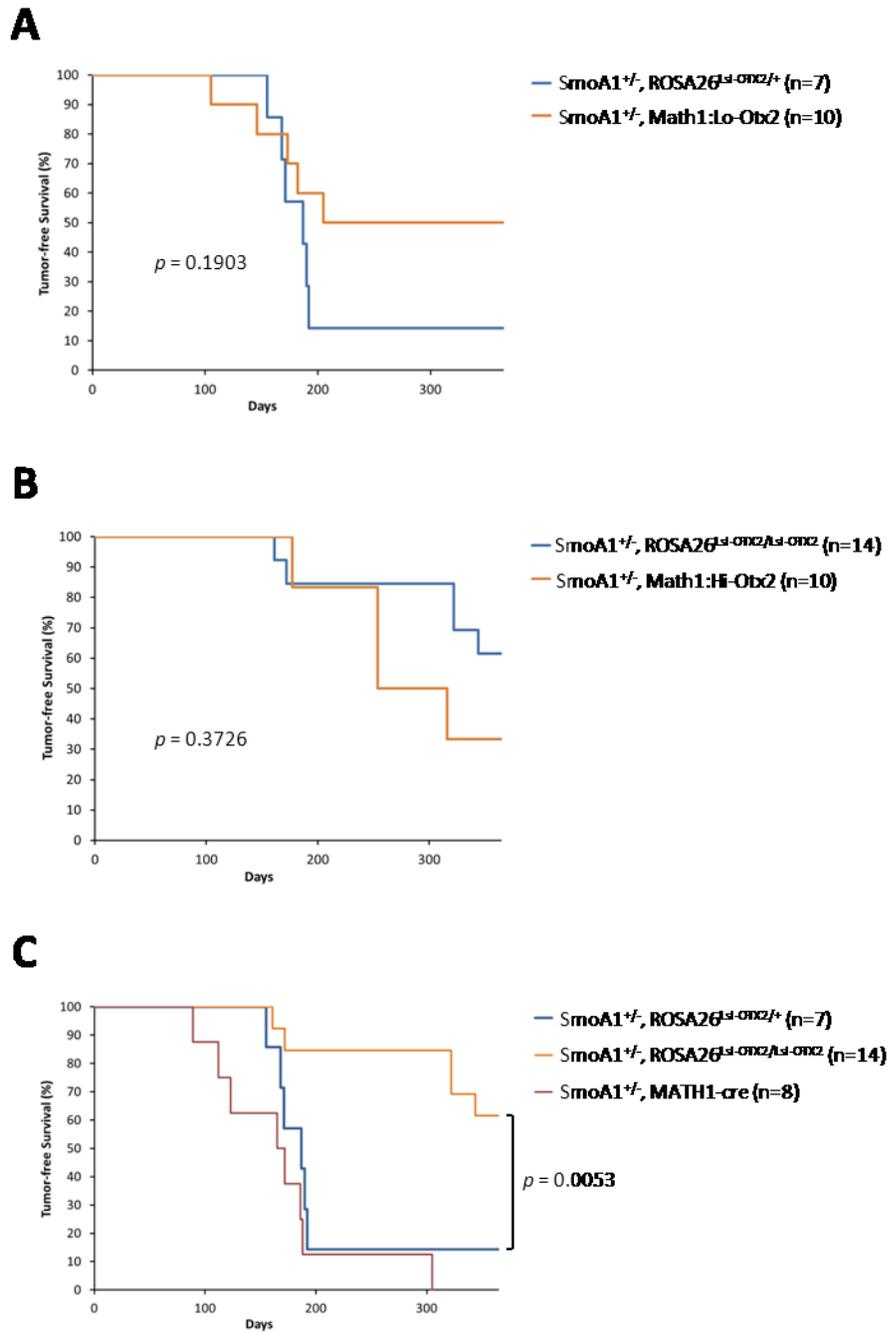
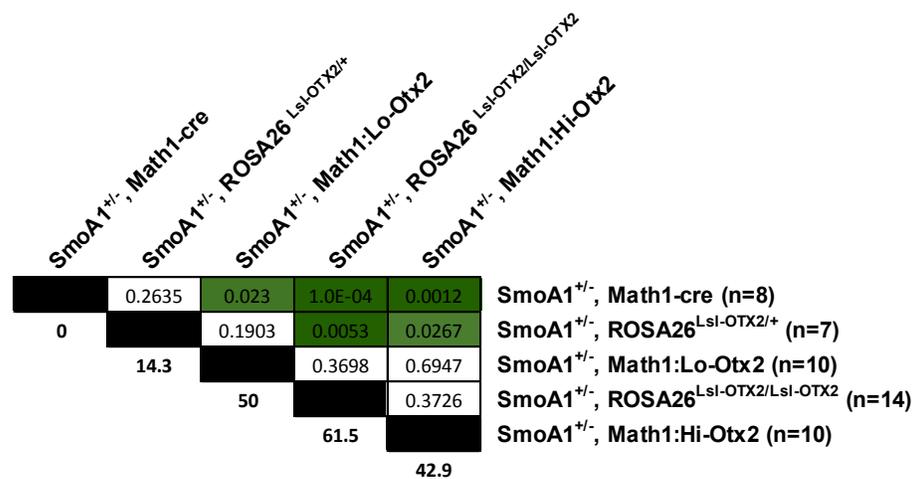


Figure 44: Tumor latency in ND2:SmoA1<sup>+/-</sup> mice expressing low and high levels of Otx2

Animals of the indicated genotypes were monitored regularly for symptoms of medulloblastoma and sacrificed when symptomatic. All medulloblastomas were histologically verified.



**Figure 45: Pairwise survival comparisons between ND2:*SmoA1*<sup>+/-</sup> animals expressing various levels of *Otx2***

Log rank test *p*-values of tumor-free survival are presented for pairwise comparisons of the indicated genotypes. Animals were monitored for symptoms of brain tumors and sacrificed when symptomatic, and medulloblastoma was then confirmed histologically. Percentage of animals of each genotype (as indicated at the top of each column) surviving for one year is indicated at the bottom of each column.

Further studies will be required to define the effects of disrupting the *ROSA26* locus or expressing *neo* upon tumorigenesis in mouse models; however it should be noted that other tumor-prone animal models have been generated from mice homozygous for *ROSA26* insertions containing the *neo* gene (Murphy et al. 2008). Regardless, the effect of high *Otx2* upon tumorigenesis in the *ND2:SmoA1*<sup>+/-</sup> model is encouraging with regards to the potential functional contribution of this gene to intermediate stages of medulloblastoma tumorigenesis.

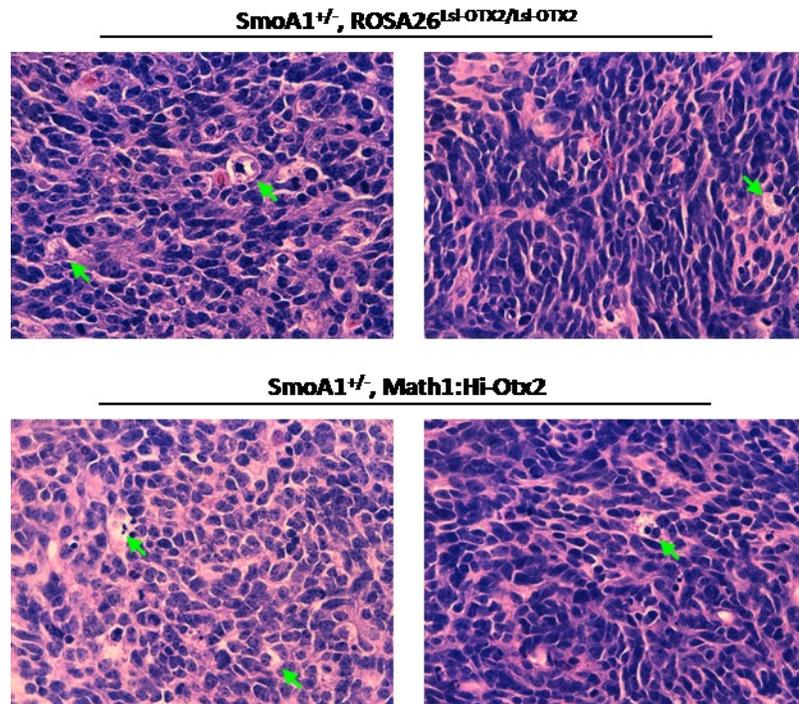


Figure 46: Histology of medulloblastomas from *SmoA1<sup>+/-</sup>, Math1:Hi-Otx2* and control mice.

Representative images of H & E stained tumors harvested from mice of the indicated genotype. All fields shown at 63x magnification. Green arrows indicate apoptotic cells.

## **5. Conclusions and future directions**

### ***5.1 OTX2 genetic alterations, overexpression, and its role in tumor maintenance***

#### **5.1.1 Spectrum of *OTX2* genetic alterations and overexpression across medulloblastoma subtypes**

Our studies have revealed various roles for *OTX2* in medulloblastoma tumorigenesis and tumor maintenance, and have generated a model of *OTX2* dysregulation in the formation of this tumor from the developing hindbrain. Our initial work focused upon the extent of *OTX2* dysregulation in medulloblastoma and its role in tumor maintenance. Although copy number alterations are typically rare genetic alterations, gain of *OTX2* occurs at a high frequency (21%) in medulloblastoma (Adamson et al. 2010), strongly implicating this gene in the pathogenesis of this tumor. Driver oncogenes are presumably overexpressed as a consequence of copy number gains, and we indeed found that *OTX2* is expressed at a high level in the majority of medulloblastomas (74%) (Adamson et al. 2010). Considering that the minimal region of overlap of the frequent chromosome 14 copy number gains in medulloblastoma contains this single gene, and that the genes adjacent to *OTX2* are not frequently expressed in medulloblastoma (Boon et al. 2002), these findings are very strong evidence that *OTX2* is the target of these alterations and suggest that its expression provides a selective advantage to emerging or established medulloblastoma. Our functional studies have revealed that all *OTX2*-expressing medulloblastoma cell lines depend upon sustained

OTX2 expression for proliferation (Adamson et al. 2010), and that *MYC* transactivation is one mechanism of OTX2-mediated tumor maintenance. Interestingly, some medulloblastomas that overexpress OTX2 do not express *MYC*, indicating that OTX2 has functions independent of *MYC* transactivation. As most medulloblastoma cell lines express high levels of *MYC* and OTX2 and thus likely represent the Group 3 molecular subtype, the establishment of Group 4 subtype experimental models should contribute substantially to the characterization of *MYC*-independent roles of OTX2.

### **5.1.2 Critical transcriptional targets of OTX2 in medulloblastoma**

OTX2 is a unique oncogene whose role in tumorigenesis has only recently been investigated. As such, various recent studies have sought to determine the target genes for this transcription factor. In our work, we identified OTX2 binding sites and regulated genes using ChIP-chip and microarray analysis to reveal the functional classes of genes regulated by OTX2. In support of our findings, various reports have identified cell cycle and neuronal development genes as OTX2 targets in the D425 medulloblastoma cell line (Bunt et al. 2011a), suggesting that OTX2 suppresses differentiation and maintains cell cycle progression in tumor cells. With the exception of *MYC*, the requirements for individual OTX2-regulated genes for the maintenance of the OTX2-mediated tumor phenotype is not known; however, the complexity of these OTX2-regulated cellular processes and the redundancies therein may reveal that few single genes are independently responsible for the effect of OTX2 upon proliferation and differentiation.

While our study found binding and regulation of *MYC* by OTX2 (Adamson et al. 2010), other studies failed to identify a transcriptional regulatory relationship between these genes (Bunt et al. 2010; Bunt et al. 2011a). These inconsistencies may reflect different OTX2 expression levels and assay conditions used in the different studies; however, both studies revealed that OTX2 is required for *MYC* protein expression, validating this regulatory relationship at some level. Uniquely, we found that OTX2 binds disproportionately to genes distinguishing medulloblastoma from normal cerebellum (Pomeroy et al. 2002). This observation is supported by genomic studies identifying enrichment for the binding motifs for CRX or OTX2 (which are nearly identical) in active transcriptional regulatory regions of highly-expressed genes in the D721 medulloblastoma cell line (Natarajan et al. 2012).

Studies of transcriptional regulation by both OTX2 and *MYC* have revealed that these factors often bind to promoters of the same genes (Bunt et al. 2011b), indicating that OTX2 regulation of *MYC* may serve as a feed-forward loop, thereby reinforcing the expression of OTX2 target genes. Such a model is supported by recent findings that *MYC* acts promiscuously to enhance existing transcriptional programs (Lin et al. 2012). Interestingly, Group 3 and 4 medulloblastomas have been proposed to represent a continuum of molecular phenotypes, with OTX2 expression being a ubiquitous feature of these tumors and *MYC* expression associating with the most aggressive subset of these tumors (Cho et al. 2011). The potential for this OTX2-*MYC* regulatory loop to

mediate tumor progression within these subgroups is an attractive yet untested possibility. Similarly, as OTX2 is not sufficient to induce *MYC* transcription in some cellular contexts, identification of the factors that drive *MYC* expression in cooperation with OTX2 would be informative regarding the critical differences between Group 3 (which express high levels of OTX2 and *MYC*) and Group 4 (OTX2<sup>+</sup>, *MYC*<sup>-</sup>) medulloblastoma. In other words, while *MYC* has been suggested to contribute to medulloblastoma progression (Stearns et al. 2006), it is unclear how *MYC* is upregulated in Group 3 tumors independent of *MYC* copy number gains.

## **5.2 The mechanisms of OTX2 dysregulation in medulloblastoma**

Understanding the mechanisms whereby oncogenes are overexpressed would reveal avenues for therapeutic interventions by inhibiting expression (and thus activity) of oncogenes that are not amenable to inhibition at the protein level. Proof of principle for this concept is reflected in studies of small molecule-mediated transcriptional repression of *MYC* (Delmore et al. 2011) and *OTX2* (Di et al. 2005). Additionally, the discovery of pathways commonly altered in tumors has potential to facilitate the design of rational therapeutic interventions. Targeting pathways (rather than individual gene products) that are critical for tumor maintenance could better account for the heterogeneity found in tumors exhibiting alterations in various checkpoints of continuous, shared pathways (Cancer Genome Atlas Research Network, 2008) . Similarly, the construction of transcriptional regulatory networks may begin to form

such linear relationships that could ultimately be exploited in tumor subtypes that are otherwise heterogeneous.

### **5.2.1 Model for OTX2 dysregulation during medulloblastoma tumorigenesis**

In this work, we have identified that *OTX2* transcription in medulloblastoma is regulated at many stages, including that of promoter DNA methylation, chromatin accessibility of regulatory elements, and activity of *trans*-acting regulators (particularly that of OTX2 protein). The observation that at the endpoint of tumor formation, OTX2 expression is self-reinforcing and requires accessibility of proximal and distal regulatory elements, it is most likely that this regulatory state is inherited from the tumor cell of origin. The *de novo* induction of OTX2 during tumor formation would require the concerted binding of various regulatory factors to mediate chromatin accessibility and transcriptional activation of the *OTX2* gene (Song et al. 2011; Thurman et al. 2012). As we have demonstrated that OTX2 is required for activation of a distal enhancer at DHS 4, which was the only differentially-active *OTX2* enhancer in medulloblastoma, the regulatory landscape of *OTX2* found in medulloblastoma is expected to be contingent upon preexisting OTX2 protein expression. Notably, during embryonic brain development, key positively-acting medulloblastoma DHS sites of the *OTX2* locus are marked with chromatin modifications indicative of strong enhancer and promoter activity, suggesting that these regulatory elements are utilized in some OTX2-expressing

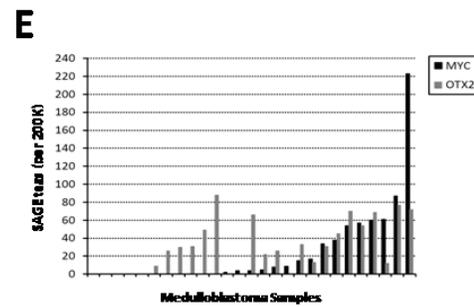
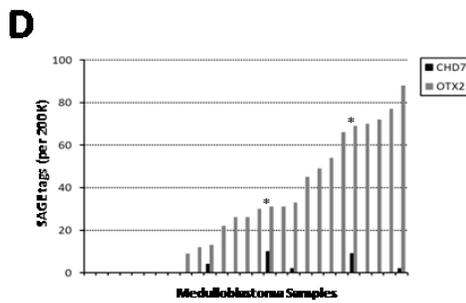
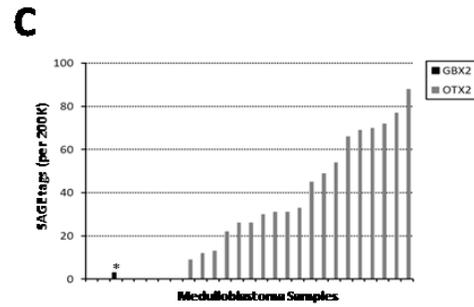
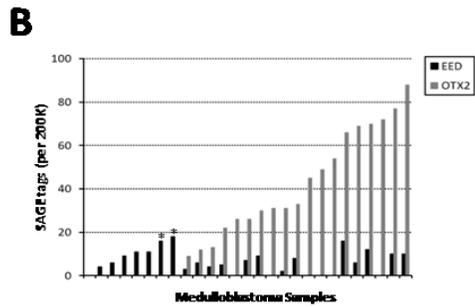
cell type in the developing brain. Given our current model, these cells likely serve as the cellular origins of OTX2-expressing medulloblastoma.

Our findings that OTX2 expression in medulloblastoma is likely to be inherited from the tumor cell of origin implies that the failure to suppress OTX2 during normal development underlies its expression in tumors. It has been described elsewhere that *Fgf8* and *Gbx2* suppress *Otx2* in the hindbrain (Li et al. 2002; Sato and Joyner 2009). Additionally, various other genes have been implicated in *Otx2* regulation at distinct domains and developmental stages (Schumacher et al. 1996; Carver et al. 2001; Kurokawa et al. 2004a; Hsiao et al. 2007; Hurd et al. 2007; Pasini et al. 2007; Takasaki et al. 2007; Sharov et al. 2008; Jia et al. 2009; Corbo et al. 2010; Muranishi et al. 2011; Inoue et al. 2012). However, none of the previously-described developmental regulators clearly associate with *Otx2* expression in established tumors (Figure 46), indicating that the nature of *Otx2* regulation is either transient (e.g. operative during tumor initiation but not intact in established tumors) or involves alternative repressors. For example, deleting *Gbx2* in the embryonic hindbrain using *En1-cre* gives rise to *Otx2*-expressing cerebellar ectopia (Li et al. 2002). The lineal relationship between these ectopia (which resemble midbrain neurons) and cerebellar ectopia of GFAP:Hi-*Otx2* mice (which resemble GNPs) is yet to be determined, though a role for *Gbx2* in suppressive aberrant proliferation through its effects on *Otx2* is encouraging with regard to our current

findings of Otx2 transcriptional regulation and the consequences of its overexpression *in vivo*.

**A**

Candidate Regulator	Direction	Domain	<i>cis</i> -element	Family	Reference	OTX2-positive v negative t-test	Spearman's rho (p-value)
Chd7*	Activator	Inner Ear	ND	ATP-dependent helicase	Hurd et al. 2010	0.203	0.30 (0.013)
Oct4/Pou5f1	Activator	ES cells	ND	POU Homeodomain	Sharov et al. 2008	0.524	0.19 (0.011)
Rax	Activator	Eye	EYE	Paired-type homeodomain	Muranishi et al. 2011	0.968	0.031 (0.051)
Lef1*	Activator	FB/MID	FM1	HMG domain (Wnt effector)	Kurokawa et al. 2004a	0.994	0.012 (0.14)
Yy1	Activator	AN	AN & V/M/C	GLI-Krüppel	Takasaki et al. 2007	0.643	0.24 (0.20)
Brn2	Activator	FB/MID	FM1	POU Homeodomain	Inoue et al. 2012	0.618+	-0.17 (0.49)+
Tcf7*	Activator	FB/MID	FM1	HMG domain (Wnt effector)	Kurokawa et al. 2004a	0.103+	-0.34 (0.43)+
Sox2	Activator	ES cells	ND	Sry Box HMG	Sharov et al. 2008	< 0.001+	-0.25 (0.27)+
Smad3*	Activator	AN	ND	Receptor-regulated Smad	Jia et al. 2009	0.0495+	-0.21 (.29)+
Brn1	Activator	FB/MID	FM1	POU Homeodomain	Inoue et al. 2012	NA	0 (0.0088)
Brn4	Activator	FB/MID	FM1	POU Homeodomain	Inoue et al. 2012	NA	0 (0.0088)
Gbx2	Repressor	Hindbrain	FM1	Homeodomain	Inoue et al. 2012	0.125	-0.24 (0.051)
Eed*	Repressor	Posterior Embryo	ND	Polycomb Group	Schumacher et al. 1996	0.0640	-0.14 (0.56)
Crx	Repressor	Eye	DHS "R"	OTX-like homeobox	Hsiau et al. 2007, Corbo et al. 2010	0.161+	0.24 (0.19)+
Hes1*	Repressor	Eye	EYE	Hes family (Notch effector)	Muranishi et al. 2011	0.534	0.0026 (0.26)+
Suz12*	Repressor	ES Cells	ND	Polycomb Group	Pasini et al. 2007	0.151+	0.32 (0.039)+
Snai1	Repressor	Posterior Embryo	ND	Zinc Finger	Carver et al. 2001	NA	0 (0.0088)



**Figure 47 (continued): Expression of developmental OTX2 regulators in medulloblastoma**

(A) Association between OTX2 expression and that of known developmental OTX2 regulators in a panel of medulloblastomas as determined by SAGE (Boon et al. 2002). \*Previously implicated in medulloblastoma, references: Chd7 (Robinson et al. 2012), Lef1/Tcf7 (Northcott et al. 2011), Smad3 (Northcott et al. 2012), Eed (Robinson et al. 2012), Hes1 (Hallahan et al. 2004), and Suz12 (Robinson et al. 2012). †Association is reversed relative to expected expression pattern (e.g. for activator or repressor function). *cis*-elements refer to elements shown in Figure 22. (B-C) Expression of most significantly OTX2-correlated genes (or negatively-correlated in the case of repressor genes) in medulloblastoma indicates these statistical differences are largely driven by outliers, as labeled with asterisks. (D) Strong association between OTX2 and MYC expression is shown as an example of a gene exhibiting a known regulatory relationship and strong correlation with OTX2.

### **5.2.2 The role of CRX in retinoid-mediated repression of OTX2**

The construction of an OTX2 regulatory pathway has potential to inform the design of OTX2-targeted therapeutics against medulloblastoma. However, the identification of the OTX2 autoregulatory loop in medulloblastoma is not clearly exploitable for therapeutic targeting, as it does not reveal additional factors other than OTX2 itself in the positive regulation of this key oncogene. However, our findings that CRX serves as a transcriptional repressor that links retinoic acid signaling to OTX2 repression is relevant to a currently-proposed medulloblastoma therapy. It has been appreciated for some time that medulloblastoma is responsive to retinoic acid treatment (Hallahan et al. 2003). Retinoic acid is a differentiation agent that exhibits activity against a broad spectrum of tumor types; generally through a diversity of signaling pathways and cellular responses (reviewed in: Wortham and Yan 2009). In medulloblastoma, for

example, retinoids affect the GNP-like differentiation state in Shh group medulloblastomas (Zhao et al. 2008) but affect non-Shh medulloblastomas by silencing OTX2 [(Bai et al. 2010) and Figure 6] and inducing bone morphogenic proteins (Hallahan et al. 2003), which themselves serve as differentiation agents. Although these responses are robust *in vitro*, preclinical studies applying retinoids to solid medulloblastomas have revealed minimal effects on xenografted tumors grown in the flank (Spiller et al. 2008) or intracranially (Bai et al. 2010). Consequently, an improved understanding of the mechanisms of retinoid-induced growth suppression in medulloblastoma could help to clarify the contradictory findings from *in vitro* and *in vivo* experiments. If retinoic acid resistance occurs *in vivo* through a failure to repress OTX2, it is possible that defective CRX induction plays some role, and thus identifying strategies of enhancing or reinforcing CRX induction during retinoic acid treatment could circumvent *in vivo* retinoid resistance. Additionally, future work should address the consequences of CRX upregulation alone in medulloblastoma, as such studies could reveal additional vulnerabilities of these tumors.

### **5.2.3 Cell populations in the developing brain utilizing the medulloblastoma-specific OTX2 enhancer**

Given our observations that a regulatory element present at DHS 4 is distinctly active in OTX2-expressing medulloblastoma, it would be of interest to define the cell types of the developing hindbrain that utilize this enhancer element. Mapping chromatin modifications of the developing brain (including the forebrain, midbrain, and

hindbrain) indicates that some cell population of this tissue exhibits enhancer marks at a region homologous to DHS 4. Although we have revealed that this enhancer is regulated by OTX2 protein, it is not utilized by all cell types that express OTX2 (such as ES cells, see Figure 10), indicating that its activity requires additional positive regulators and will not overlap completely with OTX2 expression *in vivo*.

Considering our observations of OTX2 regulation in the embryonic brain and medulloblastoma, identification of the cells utilizing this enhancer element during proper development could identify the cell types potentially giving rise to these tumors. As there are currently no genetic models of Group 3 and 4 medulloblastoma, the cellular origins of these tumors are unclear. The generation of DHS 4 reporter animals (e.g. transgenic mice expressing  $\beta$ -galactosidase driven by an enhancer of interest) would reveal specific cell types exhibiting similar *OTX2* regulation as medulloblastoma. Such a study would additionally distinguish such cells from those that express OTX2 via alternative enhancer elements. Ultimately, the ability of these cells to give rise to medulloblastoma could be tested by modeling Group 3 and 4 genetic alterations (such as MYC/MYCN overexpression or others discussed in Chapter 5.3.2 below) within these cell populations using transgenic mice expressing cre under control of the DHS 4 enhancer.

### ***5.3 The effect of aberrant Otx2 expression upon development of the hindbrain and its relationship to medulloblastoma tumorigenesis***

#### **5.3.1 Potential mechanisms of Otx2-induced migration and ectopic proliferation of hindbrain neuronal progenitor cells**

In this work, we have characterized the functional consequences of broad Otx2 overexpression upon development of the cerebellum and brainstem. While Otx2 has been studied in established medulloblastomas for some time, the effects of Otx2 overexpression in the anatomical location of medulloblastoma formation has not been previously investigated. We have uncovered a role for aberrant Otx2 in altering the spatiotemporal restrictions upon neuronal progenitor cell proliferation in the developing hindbrain. While neuronal progenitor cells of the cerebellum and brainstem normally proliferate at well-defined mitogenic niches at the EGL (Choi et al. 2005) and lower rhombic lip (Altman and Bayer 1997), respectively, aberrant Otx2 expression in these cell types gave rise to proliferation outside of these niches. Additionally, as differentiation signals outside of the mitogenic niches play some role in limiting proliferation of hindbrain neuronal progenitor cells (Choi et al. 2005), ectopic Otx2 expression circumvents such growth-inhibitory signals. Finally, Otx2 disrupts the appropriate migration of these cell populations from their mitogenic niches to their final destinations in the cerebellar IGL and at various discrete locations of the brainstem. As such, Otx2 confers various characteristics of cancer (Hanahan and Weinberg 2000) upon postnatal hindbrain progenitor cells, including growth factor-independent proliferation,

circumvention of growth inhibitory signals, and altered migration characteristics (which may have some relevance to metastatic capacity in established tumors). Although the mechanisms whereby Otx2 mediates these effects is currently unclear, in established medulloblastomas Otx2 directly regulates cell cycle genes [Table 1 and (Bunt et al. 2010; Bunt et al. 2011a)], which could contribute to the behaviors of neuronal progenitor cells in GFAP:Hi-Otx2 mice. Such relationships are underscored by the phenotypes of *Rb<sup>fl/fl</sup>* *hGFAP-cre* mice (Shakhova et al. 2006) and *p27<sup>-/-</sup>* mice (Miyazawa et al. 2000). As we did not consistently detect MYC upregulation in cerebella of GFAP:Hi-Otx2 mice (data not shown), the role of MYC in mediating the observed phenotypes of these mice is currently unclear. The effect of Otx2 upon migration of neuronal progenitors or medulloblastoma cells has not been previously-described. Notably, we did observe enrichment of genes involved in cell migration and cell motility bound and transactivated by OTX2 (Table 1), which may have some mechanistic relevance to the phenotypes observed in untransformed hindbrain cells of GFAP:Hi-Otx2 mice. The normal migration of GNPs into the IGL is mediated by the chemokine pathways transduced by the SDF-1 receptor CXCR4 (Zou et al. 1998) as well as by the Netrin receptor Unc5c (Kuramoto et al. 2004). Interestingly, *CXCR4* or *Unc5c* knockout mice exhibit progenitor cell migration phenotypes in both the cerebellum and brainstem (Zhu et al. 2009; Kim and Ackerman 2011) much like those observed in GFAP:Hi-Otx2 mice. Experimental demonstration of the relationship between Otx2 and these chemokine

signaling pathways *in vivo* should be pursued in future work. Additionally, the identification of novel targets of Otx2 when it is ectopically expressed in the hindbrain, for example by ChIP-seq analysis, would be informative regarding the mechanisms of Otx2-induced ectopia formation in these mice. The effect of Otx2 upon cell autonomous regulators of neuronal progenitor cell proliferation, in contrast to effects upon the microenvironment, is also yet to be characterized. Determining the ability of neuronal progenitor cells of GFAP:Hi-Otx2 mice to proliferate aberrantly in the context of the wild type cerebellar microenvironment, for example, following transplantation or in cerebellar slice culture, would clarify the nature of the observed effects of Otx2.

### **5.3.2 Alternative and additional strategies of deriving Otx2-initiated medulloblastomas**

Although we observed a phenotype reminiscent of medulloblastoma precursor lesions (Oliver et al. 2005; Gibson et al. 2010), the relationship between these cells and medulloblastoma have not been verified. Such studies would require the derivation of full-blown tumors from the GFAP:Hi-Otx2 mice and subsequent validation that these tumors were derived from the cerebellar or brainstem ectopia described herein.

Considering our observation that Otx2-induced ectopia differentiated as the animals reached adulthood and that the duration of proliferation for ectopia was comparable to that of normal GNPs, it is likely that intrinsic regulators of cellular lifespan limit the ability of these cells to be fully transformed by Otx2. Complete transformation may then additionally require disruption of known mediators of GNP cell cycle exit and

differentiation. For example, combining Otx2 overexpression with deletion of *Rb* or *P27*, which are known to limit GNP proliferation as they migrate away from the EGL (Miyazawa et al. 2000; Shakhova et al. 2006), may exhibit synergistic effects with Otx2. Additionally, as Otx2 is known to induce senescence in some cellular contexts (Bunt et al. 2010), the CDK inhibitor classically associated with senescence, *p21*, could be deleted to determine the ability of Otx2 to give rise to tumors when oncogene-induced senescence is circumvented. However, it should be noted that we did not observe highly-penetrant medulloblastoma from a combination of Otx2 expression and *P53* deletion, yet *p53* is also known to mediate oncogene-induced fail-safe mechanisms in some contexts (Serrano et al. 1997) including medulloblastoma formation (Pei et al. 2012).

An additional strategy of engineering tumors is to model genetic alterations known to co-occur in patient samples. Although we have modeled frequent low-level copy number alterations of *PTEN* and *P53* that are known to co-occur with *OTX2* copy number gain, targets of genetic alteration in *OTX2*-expressing tumors continue to be revealed. Such studies have identified copy number alterations or mutations of the epigenetic modifiers *CHD7*, *EZH2*, *MLL2/3*, and *UTX* in Group 3 and 4 medulloblastomas (Jones et al. 2012; Pugh et al. 2012; Robinson et al. 2012). Whether these alterations directly influence *OTX2* expression status, or rather give rise to cellular phenotypes complementary to those of *OTX2* overexpression, is yet to be determined. Mutations that co-occur with *OTX2* gains are rare; however, loss-of-function mutations

of the RNA helicase *DDX31* were recently identified to co-occur in a substantial proportion (3/7) of tumors exhibiting *OTX2* copy number gain (Pugh et al. 2012), implicating this event as being potentially cooperative with *OTX2* overexpression. The functional validation that any of these genetic alterations indeed cooperate with *OTX2* when incorporated into the GFAP:Hi-Otx2 model is yet to be determined and should be the subject of future studies.

It should be noted that our strategy of overexpressing Otx2 has some technical limitations that could affect the interpretation of this phenotype. First, it is unclear if the expression level of Otx2 is sufficiently high in this model so as to mimic the overexpression observed in medulloblastoma. Using normal cerebellum as a reference, GFAP:Hi-Otx2 animals exhibit ~10-fold induction of Otx2 (Figure 28). While some medulloblastomas express a similar level of *OTX2* mRNA relative to normal cerebellum, over 100-fold overexpression has been described in some cell lines (Di et al. 2005). Thus, a system that expresses higher levels of Otx2 by transgenic overexpression or viral transduction of Otx2 cDNA could test the ability of increased Otx2 expression to transform hindbrain cell types. Finally, although the *hGFAP-cre* transgene drives broad Otx2 expression in our current mouse model, it is possible that the true cell of origin for Otx2-expressing medulloblastoma has already been generated by the time recombination occurs in these animals. For example, progenitors of cerebellar Purkinje cells do not undergo robust recombination using the *hGFAP-cre* transgene (Yang et al.

2008), and the potential for these cells to give rise to non-Shh medulloblastoma is unknown. Thus, it would be of interest to attempt to derive medulloblastomas using alternative transgenes engineered to express cre at earlier developmental timepoints or in specific cells predicted to give rise to Group 3 or 4 medulloblastomas based on gene expression patterns (as exemplified by Gibson et al. 2010) or other characteristics of these tumors (see Chapters 5.2.3 and 5.4).

#### **5.4 Implications of OTX2 autoregulation for the phenotype observed in GFAP:Hi-Otx2 mice**

Our studies have described the mechanisms of OTX2 dysregulation in medulloblastoma as well as the effects of OTX2 overexpression on the developing mouse hindbrain. In the case that the ectopia that accumulate in GFAP:Hi-Otx2 mice truly represent a medulloblastoma precursor lesion, which is yet to be determined, the genomic and *in vivo* studies described herein would reveal some complementary observations. While we described that OTX2 expression is likely to be inherited from the tumor cell of origin based on its observed transcriptional regulation in established medulloblastomas, it follows that the phenotype observed in GFAP:Hi-Otx2 mice could occur due to the failure of some OTX2-expressing cell to repress OTX2 over the course of normal hindbrain development. Our experiments with different cre drivers also suggest that this phenotype relies on Otx2 induction at an early stage of GNP specification (or prior to GNP specification). Otx2 expression has been identified in portions of the rhombic lip (Frantz et al. 1994) but not in the cerebellar ventricular zone (Jones et al.

2009). The rhombic lip does not express cre protein in Math1-cre animals (Yang et al. 2008), and thus in Math1:Hi-Otx2 mice, Otx2 expression would be initiated at the EGL rather than the rhombic lip. If ectopia of GFAP:Hi-Otx2 mice form due to a failure to repress Otx2 through a normal developmental program, Otx2 would most likely be inherited from newly-specified GNPs of the lateral rhombic lip that naturally express Otx2 (Jones et al. 2009). This hypothesis could be experimentally addressed using *Otx2-creER* mice (Fossat et al. 2006) to enforce Otx2 expression in these cells.

## **5.5 Summary and closing remarks**

Our studies have comprehensively investigated the mechanisms of OTX2 dysregulation and its functional consequences in medulloblastomas and its anatomical origins. This work has revealed stark differences between the transcriptional and genomic landscape of the *OTX2* gene among distinct medulloblastoma subgroups. Additionally, we have revealed cellular phenotypes reminiscent of cancer hallmarks induced by Otx2 in normal hindbrain cells. Although we have yet to establish an animal model of Otx2-driven medulloblastoma, these studies establish a framework for the assessment of cooperating genetic alterations in promoting medulloblastoma development from aberrantly-proliferating cells described herein. We foresee great strides being made by applying the findings of medulloblastoma genomic studies to the modeling of these alterations in the developing hindbrain alone and in combination with Otx2.

## Appendix

Figures and tables from Chapter 2, with the exceptions of Figures 6 and 7 and Tables 1 and 2, were adapted (or reprinted) by permission from the American Association for Cancer Research: Adamson DC, Shi Q, Wortham M, Northcott PA, Di C, Duncan CG, Li J, McLendon RE, Bigner DD, Taylor MD and Yan H, OTX2 is critical for the maintenance and progression of Shh-independent medulloblastomas, *Cancer Research*, 2010, 70(1), 181-191. Figures from Chapter 4, which were originally published in (Wortham et al. 2012) are covered by the creative Commons License, under which authors retain copyright ownership for published work as according to *PLoS* policy.

## References

- Acampora D, Avantaggiato V, Tuorto F, Simeone A. 1997. Genetic control of brain morphogenesis through Otx gene dosage requirement. *Development* **124**(18): 3639-3650.
- Adamson DC, Shi Q, Wortham M, Northcott PA, Di C, Duncan CG, Li J, McLendon RE, Bigner DD, Taylor MD et al. 2010. OTX2 is critical for the maintenance and progression of Shh-independent medulloblastomas. *Cancer Res* **70**(1): 181-191.
- Altman J, Bayer SA. 1997. *Development of the cerebellar system: in relation to its evolution, structure, and function*. CRC Press, New York.
- Bai R, Siu IM, Tyler BM, Staedtke V, Gallia GL, Riggins GJ. 2010. Evaluation of retinoic acid therapy for OTX2-positive medulloblastomas. *Neuro Oncol* **12**(7): 655-663.
- Beissbarth T, Speed TP. 2004. Gostat: find statistically overrepresented Gene Ontologies within a group of genes. *Bioinformatics* **20**(9): 1464-1465.
- Boon K, Eberhart CG, Riggins GJ. 2005. Genomic amplification of orthodenticle homologue 2 in medulloblastomas. *Cancer Res* **65**(3): 703-707.
- Boon K, Osorio EC, Greenhut SF, Schaefer CF, Shoemaker J, Polyak K, Morin PJ, Buetow KH, Strausberg RL, De Souza SJ et al. 2002. An anatomy of normal and malignant gene expression. *Proc Natl Acad Sci U S A* **99**(17): 11287-11292.
- Boyle AP, Guinney J, Crawford GE, Furey TS. 2008. F-Seq: a feature density estimator for high-throughput sequence tags. *Bioinformatics* **24**(21): 2537-2538.
- Broccoli V, Boncinelli E, Wurst W. 1999. The caudal limit of Otx2 expression positions the isthmic organizer. *Nature* **401**(6749): 164-168.
- Bryne JC, Valen E, Tang MH, Marstrand T, Winther O, da Piedade I, Krogh A, Lenhard B, Sandelin A. 2008. JASPAR, the open access database of transcription factor-binding profiles: new content and tools in the 2008 update. *Nucleic Acids Res* **36**(Database issue): D102-106.
- Bunt J, de Haas TG, Hasselt NE, Zwijnenburg DA, Koster J, Versteeg R, Kool M. 2010. Regulation of cell cycle genes and induction of senescence by overexpression of OTX2 in medulloblastoma cell lines. *Mol Cancer Res* **8**(10): 1344-1357.

- Bunt J, Hasselt NE, Zwijnenburg DA, Hamdi M, Koster J, Versteeg R, Kool M. 2011a. OTX2 directly activates cell cycle genes and inhibits differentiation in medulloblastoma cells. *Int J Cancer* **131**(2):E21-32.
- Bunt J, Hasselt NE, Zwijnenburg DA, Koster J, Versteeg R, Kool M. 2011b. Joint binding of OTX2 and MYC in promoter regions is associated with high gene expression in medulloblastoma. *PLoS One* **6**(10): e26058.
- Carver EA, Jiang R, Lan Y, Oram KF, Gridley T. 2001. The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol Cell Biol* **21**(23): 8184-8188.
- Castellino RC, Barwick BG, Schniederjan M, Buss MC, Becher O, Hambarzumyan D, Macdonald TJ, Brat DJ, Durden DL. 2010. Heterozygosity for Pten promotes tumorigenesis in a mouse model of medulloblastoma. *PLoS One* **5**(5): e10849.
- Cho YJ, Tsherniak A, Tamayo P, Santagata S, Ligon A, Greulich H, Berhoukim R, Amani V, Goumnerova L, Eberhart CG et al. 2011. Integrative genomic analysis of medulloblastoma identifies a molecular subgroup that drives poor clinical outcome. *J Clin Oncol* **29**(11): 1424-1430.
- Choi Y, Borghesani PR, Chan JA, Segal RA. 2005. Migration from a mitogenic niche promotes cell-cycle exit. *J Neurosci* **25**(45): 10437-10445.
- Corbo JC, Lawrence KA, Karlstetter M, Myers CA, Abdelaziz M, Dirkes W, Weigelt K, Seifert M, Benes V, Fritsche LG et al. 2010. CRX ChIP-seq reveals the cis-regulatory architecture of mouse photoreceptors. *Genome Res* **20**(11): 1512-1525.
- Crawford GE, Holt IE, Mullikin JC, Tai D, Blakesley R, Bouffard G, Young A, Masiello C, Green ED, Wolfsberg TG et al. 2004. Identifying gene regulatory elements by genome-wide recovery of DNase hypersensitive sites. *Proc Natl Acad Sci U S A* **101**(4): 992-997.
- Crawford GE, Holt IE, Whittle J, Webb BD, Tai D, Davis S, Margulies EH, Chen Y, Bernat JA, Ginsburg D et al. 2006. Genome-wide mapping of DNase hypersensitive sites using massively parallel signature sequencing (MPSS). *Genome Res* **16**(1): 123-131.
- Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, Kastiris E, Gilpatrick T, Paranal RM, Qi J et al. 2011. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell* **146**(6): 904-917.

- Dey J, Ditzler S, Knoblauch SE, Hatton BA, Schelter JM, Cleary MA, Mecham B, Rorke-Adams LB, Olson JM. 2012. A distinct smoothed mutation causes severe cerebellar developmental defects and medulloblastoma in a novel transgenic mouse model. *Mol Cell Biol* **32**(20): 4104-4115.
- Di C, Liao S, Adamson DC, Parrett TJ, Broderick DK, Shi Q, Lengauer C, Cummins JM, Velculescu VE, Fufts DW et al. 2005. Identification of OTX2 as a medulloblastoma oncogene whose product can be targeted by all-trans retinoic acid. *Cancer Res* **65**(3): 919-924.
- Eberhart CG. 2012. Three down and one to go: modeling medulloblastoma subgroups. *Cancer Cell* **21**(2): 137-138.
- Eberhart CG, Burger PC. 2003. Anaplasia and grading in medulloblastomas. *Brain Pathol* **13**(3): 376-385.
- Emerson MM, Cepko CL. 2011. Identification of a retina-specific Otx2 enhancer element active in immature developing photoreceptors. *Dev Biol* **360**(1): 241-255.
- Ernst J, Kheradpour P, Mikkelson TS, Shores N, Ward LD, Epstein CB, Zhang X, Wang L, Issner R, Coyne M et al. 2011. Mapping and analysis of chromatin state dynamics in nine human cell types. *Nature* **473**(7345): 43-49.
- Fenaux P, Chastang C, Chomienne C, Castaigne S, Sanz M, Link H, Lowenberg B, Fey M, Archim-Baud E, Degos L et al. 1995. Treatment of newly diagnosed acute promyelocytic leukemia (APL) by all transretinoic acid (ATRA) combined with chemotherapy: The European experience. European APL Group. *Leuk Lymphoma* **16**(5-6): 431-437.
- Fossat N, Chatelain G, Brun G, Lamonerie T. 2006. Temporal and spatial delineation of mouse Otx2 functions by conditional self-knockout. *EMBO Rep* **7**(8): 824-830.
- Fossat N, Courtois V, Chatelain G, Brun G, Lamonerie T. 2005. Alternative usage of Otx2 promoters during mouse development. *Dev Dyn* **233**(1): 154-160.
- Frantz GD, Weimann JM, Levin ME, McConnell SK. 1994. Otx1 and Otx2 define layers and regions in developing cerebral cortex and cerebellum. *J Neurosci* **14**(10): 5725-5740.
- Frappart PO, Lee Y, Russell HR, Chalhoub N, Wang YD, Orii KE, Zhao J, Kondo N, Baker SJ, McKinnon PJ. 2009. Recurrent genomic alterations characterize

- medulloblastoma arising from DNA double-strand break repair deficiency. *Proc Natl Acad Sci U S A* **106**(6): 1880-1885.
- Fults D, Pedone C, Dai C, Holland EC. 2002. MYC expression promotes the proliferation of neural progenitor cells in culture and in vivo. *Neoplasia* **4**(1): 32-39.
- Gajjar A, Hernan R, Kocak M, Fuller C, Lee Y, McKinnon PJ, Wallace D, Lau C, Chintagumpala M, Ashley DM et al. 2004. Clinical, histopathologic, and molecular markers of prognosis: toward a new disease risk stratification system for medulloblastoma. *J Clin Oncol* **22**(6): 984-993.
- Gibson P, Tong Y, Robinson G, Thompson MC, Currle DS, Eden C, Kranenburg TA, Hogg T, Poppleton H, Martin J et al. 2010. Subtypes of medulloblastoma have distinct developmental origins. *Nature* **468**(7327): 1095-1099.
- Goodrich LV, Milenkovic L, Higgins KM, Scott MP. 1997. Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* **277**(5329): 1109-1113.
- Grammel D, Warmuth-Metz M, von Bueren AO, Kool M, Pietsch T, Kretzschmar HA, Rowitch DH, Rutkowski S, Pfister SM, Schuller U. 2012. Sonic hedgehog-associated medulloblastoma arising from the cochlear nuclei of the brainstem. *Acta Neuropathol* **123**(4): 601-614.
- Haeussler M, Joly JS. 2011. When needles look like hay: how to find tissue-specific enhancers in model organism genomes. *Dev Biol* **350**(2): 239-254.
- Hallahan AR, Pritchard JI, Chandraratna RA, Ellenbogen RG, Geyer JR, Overland RP, Strand AD, Tapscott SJ, Olson JM. 2003. BMP-2 mediates retinoid-induced apoptosis in medulloblastoma cells through a paracrine effect. *Nat Med* **9**(8): 1033-1038.
- Hallahan AR, Pritchard JI, Hansen S, Benson M, Stoeck J, Hatton BA, Russell TL, Ellenbogen RG, Bernstein ID, Beachy PA et al. 2004. The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas. *Cancer Res* **64**(21): 7794-7800.
- Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. *Cell* **100**(1): 57-70.
- He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. 1998. Identification of c-MYC as a target of the APC pathway. *Science* **281**(5382): 1509-1512.

- Hidalgo-Sanchez M, Simeone A, Alvarado-Mallart RM. 1999. Fgf8 and Gbx2 induction concomitant with Otx2 repression is correlated with midbrain-hindbrain fate of caudal prosencephalon. *Development* **126**(14): 3191-3203.
- Hoppe-Hirsch E, Renier D, Lellouch-Tubiana A, Sainte-Rose C, Pierre-Kahn A, Hirsch JF. 1990. Medulloblastoma in childhood: progressive intellectual deterioration. *Childs Nerv Syst* **6**(2): 60-65.
- Hsiau TH, Diaconu C, Myers CA, Lee J, Cepko CL, Corbo JC. 2007. The cis-regulatory logic of the mammalian photoreceptor transcriptional network. *PLoS One* **2**(7): e643.
- Hu S, Xie Z, Onishi A, Yu X, Jiang L, Lin J, Rho HS, Woodard C, Wang H, Jeong JS et al. 2009. Profiling the human protein-DNA interactome reveals ERK2 as a transcriptional repressor of interferon signaling. *Cell* **139**(3): 610-622.
- Hurd EA, Capers PL, Blauwkamp MN, Adams ME, Raphael Y, Poucher HK, Martin DM. 2007. Loss of Chd7 function in gene-trapped reporter mice is embryonic lethal and associated with severe defects in multiple developing tissues. *Mamm Genome* **18**(2): 94-104.
- Inoue F, Kurokawa D, Takahashi M, Aizawa S. 2012. Gbx2 directly restricts Otx2 expression to forebrain and midbrain, competing with class III POU factors. *Mol Cell Biol* **32**(13): 2618-2627.
- Jia S, Wu D, Xing C, Meng A. 2009. Smad2/3 activities are required for induction and patterning of the neuroectoderm in zebrafish. *Dev Biol* **333**(2): 273-284.
- Jones AR, Overly CC, Sunkin SM. 2009. The Allen Brain Atlas: 5 years and beyond. *Nat Rev Neurosci* **10**(11): 821-828.
- Jones DT, Jager N, Kool M, Zichner T, Hutter B, Sultan M, Cho YJ, Pugh TJ, Hovestadt V, Stutz AM et al. 2012. Dissecting the genomic complexity underlying medulloblastoma. *Nature* **488**(7409): 100-105.
- Jonkers J, Meuwissen R, van der Gulden H, Peterse H, van der Valk M, Berns A. 2001. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. *Nat Genet* **29**(4): 418-425.
- Kawauchi D, Robinson G, Uziel T, Gibson P, Rehg J, Gao C, Finkelstein D, Qu C, Pounds S, Ellison DW et al. 2012. A mouse model of the most aggressive subgroup of human medulloblastoma. *Cancer Cell* **21**(2): 168-180.

- Kim D, Ackerman SL. 2011. The UNC5C netrin receptor regulates dorsal guidance of mouse hindbrain axons. *J Neurosci* **31**(6): 2167-2179.
- Kimura-Yoshida C, Kitajima K, Oda-Ishii I, Tian E, Suzuki M, Yamamoto M, Suzuki T, Kobayashi M, Aizawa S, Matsuo I. 2004. Characterization of the pufferfish Otx2 cis-regulators reveals evolutionarily conserved genetic mechanisms for vertebrate head specification. *Development* **131**(1): 57-71.
- Kleihues P, Louis DN, Scheithauer BW, Rorke LB, Reifenberger G, Burger PC, Cavenee WK. 2002. The WHO classification of tumors of the nervous system. *J Neuropathol Exp Neurol* **61**(3): 215-225; discussion 226-219.
- Kuramoto T, Kuwamura M, Serikawa T. 2004. Rat neurological mutations cerebellar vermis defect and hobble are caused by mutations in the netrin-1 receptor gene Unc5h3. *Brain Res Mol Brain Res* **122**(2): 103-108.
- Kurokawa D, Kiyonari H, Nakayama R, Kimura-Yoshida C, Matsuo I, Aizawa S. 2004a. Regulation of Otx2 expression and its functions in mouse forebrain and midbrain. *Development* **131**(14): 3319-3331.
- Kurokawa D, Takasaki N, Kiyonari H, Nakayama R, Kimura-Yoshida C, Matsuo I, Aizawa S. 2004b. Regulation of Otx2 expression and its functions in mouse epiblast and anterior neuroectoderm. *Development* **131**(14): 3307-3317.
- Lee BK, Bhinge AA, Battenhouse A, McDaniel RM, Liu Z, Song L, Ni Y, Birney E, Lieb JD, Furey TS et al. 2012. Cell-type specific and combinatorial usage of diverse transcription factors revealed by genome-wide binding studies in multiple human cells. *Genome Res* **22**(1): 9-24.
- Levine M, Tjian R. 2003. Transcription regulation and animal diversity. *Nature* **424**(6945): 147-151.
- Li JY, Lao Z, Joyner AL. 2002. Changing requirements for Gbx2 in development of the cerebellum and maintenance of the mid/hindbrain organizer. *Neuron* **36**(1): 31-43.
- Lin CY, Loven J, Rahl PB, Paranal RM, Burge CB, Bradner JE, Lee TI, Young RA. 2012. Transcriptional Amplification in Tumor Cells with Elevated c-Myc. *Cell* **151**(1): 56-67.
- Lindsey JC, Hill RM, Megahed H, Lusher ME, Schwalbe EC, Cole M, Hogg TL, Gilbertson RJ, Ellison DW, Bailey S et al. 2011. TP53 mutations in favorable-risk

- Wnt/Wingless-subtype medulloblastomas. *J Clin Oncol* **29**(12): e344-346; author reply e347-348.
- Matei V, Pauley S, Kaing S, Rowitch D, Beisel KW, Morris K, Feng F, Jones K, Lee J, Fritsch B. 2005. Smaller inner ear sensory epithelia in Neurog 1 null mice are related to earlier hair cell cycle exit. *Dev Dyn* **234**(3): 633-650.
- Matys V, Kel-Margoulis OV, Fricke E, Liebich I, Land S, Barre-Dirrie A, Reuter I, Chekmenev D, Krull M, Hornischer K et al. 2006. TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. *Nucleic Acids Res* **34**(Database issue): D108-110.
- Miyazawa K, Himi T, Garcia V, Yamagishi H, Sato S, Ishizaki Y. 2000. A role for p27/Kip1 in the control of cerebellar granule cell precursor proliferation. *J Neurosci* **20**(15): 5756-5763.
- Muranishi Y, Terada K, Inoue T, Katoh K, Tsujii T, Sanuki R, Kurokawa D, Aizawa S, Tamaki Y, Furukawa T. 2011. An essential role for RAX homeoprotein and NOTCH-HES signaling in Otx2 expression in embryonic retinal photoreceptor cell fate determination. *J Neurosci* **31**(46): 16792-16807.
- Murphy DJ, Junttila MR, Pouyet L, Karnezis A, Shchors K, Bui DA, Brown-Swigart L, Johnson L, Evan GI. 2008. Distinct thresholds govern Myc's biological output in vivo. *Cancer Cell* **14**(6): 447-457.
- Natarajan A, Yardimci GG, Sheffield NC, Crawford GE, Ohler U. 2012. Predicting cell-type-specific gene expression from regions of open chromatin. *Genome Res* **22**(9): 1711-1722.
- Neph S, Vierstra J, Stergachis AB, Reynolds AP, Haugen E, Vernot B, Thurman RE, John S, Sandstrom R, Johnson AK et al. 2012. An expansive human regulatory lexicon encoded in transcription factor footprints. *Nature* **489**(7414): 83-90.
- Northcott PA, Fernandez LA, Hagan JP, Ellison DW, Grajkowska W, Gillespie Y, Grundy R, Van Meter T, Rutka JT, Croce CM et al. 2009a. The miR-17/92 polycistron is up-regulated in sonic hedgehog-driven medulloblastomas and induced by N-myc in sonic hedgehog-treated cerebellar neural precursors. *Cancer Res* **69**(8): 3249-3255.
- Northcott PA, Korshunov A, Witt H, Hielscher T, Eberhart CG, Mack S, Bouffet E, Clifford SC, Hawkins CE, French P et al. 2011. Medulloblastoma comprises four distinct molecular variants. *J Clin Oncol* **29**(11): 1408-1414.

- Northcott PA, Nakahara Y, Wu X, Feuk L, Ellison DW, Croul S, Mack S, Kongkham PN, Peacock J, Dubuc A et al. 2009b. Multiple recurrent genetic events converge on control of histone lysine methylation in medulloblastoma. *Nat Genet* **41**(4): 465-472.
- Northcott PA, Shih DJ, Peacock J, Garzia L, Morrissy AS, Zichner T, Stutz AM, Korshunov A, Reimand J, Schumacher SE et al. 2012. Subgroup-specific structural variation across 1,000 medulloblastoma genomes. *Nature* **488**(7409): 49-56.
- Nusse R, Varmus HE. 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* **31**(1): 99-109.
- O'Hayer KM, Counter CM. 2006. A genetically defined normal human somatic cell system to study ras oncogenesis in vivo and in vitro. *Methods Enzymol* **407**: 637-647.
- Oliver TG, Read TA, Kessler JD, Mehmeti A, Wells JF, Huynh TT, Lin SM, Wechsler-Reya RJ. 2005. Loss of patched and disruption of granule cell development in a pre-neoplastic stage of medulloblastoma. *Development* **132**(10): 2425-2439.
- Omodei D, Acampora D, Mancuso P, Prakash N, Di Giovannantonio LG, Wurst W, Simeone A. 2008. Anterior-posterior graded response to Otx2 controls proliferation and differentiation of dopaminergic progenitors in the ventral mesencephalon. *Development* **135**(20): 3459-3470.
- Packer RJ, Reddy A. 2004. New Treatments in Pediatric Brain Tumors. *Curr Treat Options Neurol* **6**(5): 377-389.
- Packer RJ, Sutton LN, Goldwein JW, Perilongo G, Bunin G, Ryan J, Cohen BH, D'Angio G, Kramer ED, Zimmerman RA et al. 1991. Improved survival with the use of adjuvant chemotherapy in the treatment of medulloblastoma. *J Neurosurg* **74**(3): 433-440.
- Packer RJ, Zhou T, Holmes E, Vezina G, Gajjar A. 2012. Survival and secondary tumors in children with medulloblastoma receiving radiotherapy and adjuvant chemotherapy: results of Children's Oncology Group trial A9961. *Neuro Oncol*.
- Parsons DW, Li M, Zhang X, Jones S, Leary RJ, Lin JC, Boca SM, Carter H, Samayoa J, Bettegowda C et al. 2011. The genetic landscape of the childhood cancer medulloblastoma. *Science* **331**(6016): 435-439.

- Pasini D, Bracken AP, Hansen JB, Capillo M, Helin K. 2007. The polycomb group protein Suz12 is required for embryonic stem cell differentiation. *Mol Cell Biol* **27**(10): 3769-3779.
- Pei Y, Moore CE, Wang J, Tewari AK, Eroshkin A, Cho YJ, Witt H, Korshunov A, Read TA, Sun JL et al. 2012. An animal model of MYC-driven medulloblastoma. *Cancer Cell* **21**(2): 155-167.
- Pietsch T, Taylor MD, Rutka JT. 2004. Molecular pathogenesis of childhood brain tumors. *J Neurooncol* **70**(2): 203-215.
- Pomeroy SL, Tamayo P, Gaasenbeek M, Sturla LM, Angelo M, McLaughlin ME, Kim JY, Goumnerova LC, Black PM, Lau C et al. 2002. Prediction of central nervous system embryonal tumour outcome based on gene expression. *Nature* **415**(6870): 436-442.
- Puelles E, Acampora D, Gogoi R, Tuorto F, Papalia A, Guillemot F, Ang SL, Simeone A. 2006. Otx2 controls identity and fate of glutamatergic progenitors of the thalamus by repressing GABAergic differentiation. *J Neurosci* **26**(22): 5955-5964.
- Pugh TJ, Weeraratne SD, Archer TC, Pomeranz Krummel DA, Auclair D, Bochicchio J, Carneiro MO, Carter SL, Cibulskis K, Erlich RL et al. 2012. Medulloblastoma exome sequencing uncovers subtype-specific somatic mutations. *Nature* **488**(7409): 106-110.
- Ray A, Ho M, Ma J, Parkes RK, Mainprize TG, Ueda S, McLaughlin J, Bouffet E, Rutka JT, Hawkins CE. 2004. A clinicobiological model predicting survival in medulloblastoma. *Clin Cancer Res* **10**(22): 7613-7620.
- Read TA, Fogarty MP, Markant SL, McLendon RE, Wei Z, Ellison DW, Febbo PG, Wechsler-Reya RJ. 2009. Identification of CD15 as a marker for tumor-propagating cells in a mouse model of medulloblastoma. *Cancer Cell* **15**(2): 135-147.
- Ris MD, Packer R, Goldwein J, Jones-Wallace D, Boyett JM. 2001. Intellectual outcome after reduced-dose radiation therapy plus adjuvant chemotherapy for medulloblastoma: a Children's Cancer Group study. *J Clin Oncol* **19**(15): 3470-3476.
- Robinson G, Parker M, Kranenburg TA, Lu C, Chen X, Ding L, Phoenix TN, Hedlund E, Wei L, Zhu X et al. 2012. Novel mutations target distinct subgroups of medulloblastoma. *Nature*. **488**(7409):43-48.

- Romer JT, Kimura H, Magdaleno S, Sasai K, Fuller C, Baines H, Connelly M, Stewart CF, Gould S, Rubin LL et al. 2004. Suppression of the Shh pathway using a small molecule inhibitor eliminates medulloblastoma in Ptc1(+/-)p53(-/-) mice. *Cancer Cell* **6**(3): 229-240.
- Rudin CM, Hann CL, Laterra J, Yauch RL, Callahan CA, Fu L, Holcomb T, Stinson J, Gould SE, Coleman B et al. 2009. Treatment of medulloblastoma with hedgehog pathway inhibitor GDC-0449. *N Engl J Med* **361**(12): 1173-1178.
- Sandelin A, Wasserman WW, Lenhard B. 2004. ConSite: web-based prediction of regulatory elements using cross-species comparison. *Nucleic Acids Res* **32**(Web Server issue): W249-252.
- Sato T, Joyner AL. 2009. The duration of Fgf8 isthmic organizer expression is key to patterning different tectal-isthmo-cerebellum structures. *Development* **136**(21): 3617-3626.
- Schuller U, Heine VM, Mao J, Kho AT, Dillon AK, Han YG, Huillard E, Sun T, Ligon AH, Qian Y et al. 2008. Acquisition of granule neuron precursor identity is a critical determinant of progenitor cell competence to form Shh-induced medulloblastoma. *Cancer Cell* **14**(2): 123-134.
- Schumacher A, Faust C, Magnuson T. 1996. Positional cloning of a global regulator of anterior-posterior patterning in mice. *Nature* **384**(6610): 648.
- Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**(5): 593-602.
- Shakhova O, Leung C, van Montfort E, Berns A, Marino S. 2006. Lack of Rb and p53 delays cerebellar development and predisposes to large cell anaplastic medulloblastoma through amplification of N-Myc and Ptch2. *Cancer Res* **66**(10): 5190-5200.
- Sharov AA, Masui S, Sharova LV, Piao Y, Aiba K, Matoba R, Xin L, Niwa H, Ko MS. 2008. Identification of Pou5f1, Sox2, and Nanog downstream target genes with statistical confidence by applying a novel algorithm to time course microarray and genome-wide chromatin immunoprecipitation data. *BMC Genomics* **9**: 269.
- Shen-Ong GL, Keath EJ, Piccoli SP, Cole MD. 1982. Novel myc oncogene RNA from abortive immunoglobulin-gene recombination in mouse plasmacytomas. *Cell* **31**(2 Pt 1): 443-452.

- Simeone A, Acampora D, Mallamaci A, Stornaiuolo A, D'Apice MR, Nigro V, Boncinelli E. 1993. A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. *EMBO J* **12**(7): 2735-2747.
- Simeone A, Avantaggiato V, Moroni MC, Mavilio F, Arra C, Cotelli F, Nigro V, Acampora D. 1995. Retinoic acid induces stage-specific antero-posterior transformation of rostral central nervous system. *Mech Dev* **51**(1): 83-98.
- Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB. 2004. Identification of human brain tumour initiating cells. *Nature* **432**(7015): 396-401.
- Song JS, Johnson WE, Zhu X, Zhang X, Li W, Manrai AK, Liu JS, Chen R, Liu XS. 2007. Model-based analysis of two-color arrays (MA2C). *Genome Biol* **8**(8): R178.
- Song L, Zhang Z, Graseder LL, Boyle AP, Giresi PG, Lee BK, Sheffield NC, Graf S, Huss M, Keefe D et al. 2011. Open chromatin defined by DNaseI and FAIRE identifies regulatory elements that shape cell-type identity. *Genome Res* **21**(10): 1757-1767.
- Soriano P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* **21**(1): 70-71.
- Spiller SE, Ditzler SH, Pullar BJ, Olson JM. 2008. Response of preclinical medulloblastoma models to combination therapy with 13-cis retinoic acid and suberoylanilide hydroxamic acid (SAHA). *J Neurooncol* **87**(2): 133-141.
- Srinivas S, Watanabe T, Lin CS, Williams CM, Tanabe Y, Jessell TM, Costantini F. 2001. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* **1**: 4.
- Stavrou T, Bromley CM, Nicholson HS, Byrne J, Packer RJ, Goldstein AM, Reaman GH. 2001. Prognostic factors and secondary malignancies in childhood medulloblastoma. *J Pediatr Hematol Oncol* **23**(7): 431-436.
- Stearns D, Chaudhry A, Abel TW, Burger PC, Dang CV, Eberhart CG. 2006. c-myc overexpression causes anaplasia in medulloblastoma. *Cancer Res* **66**(2): 673-681.
- Swartling FJ, Grimmer MR, Hackett CS, Northcott PA, Fan QW, Goldenberg DD, Lau J, Masic S, Nguyen K, Yakovenko S et al. 2010. Pleiotropic role for MYCN in medulloblastoma. *Genes Dev* **24**(10): 1059-1072.

- Swartling FJ, Savov V, Persson AI, Chen J, Hackett CS, Northcott PA, Grimmer MR, Lau J, Chesler L, Perry A et al. 2012. Distinct neural stem cell populations give rise to disparate brain tumors in response to N-MYC. *Cancer Cell* **21**(5): 601-613.
- Takasaki N, Kurokawa D, Nakayama R, Nakayama J, Aizawa S. 2007. Acetylated YY1 regulates Otx2 expression in anterior neuroectoderm at two cis-sites 90 kb apart. *EMBO J* **26**(6): 1649-1659.
- Thomas KR, Capecchi MR. 1990. Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* **346**(6287): 847-850.
- Thurman RE, Rynes E, Humbert R, Vierstra J, Maurano MT, Haugen E, Sheffield NC, Stergachis AB, Wang H, Vernot B et al. 2012. The accessible chromatin landscape of the human genome. *Nature* **489**(7414): 75-82.
- Trotman LC, Niki M, Dotan ZA, Koutcher JA, Di Cristofano A, Xiao A, Khoo AS, Roy-Burman P, Greenberg NM, Van Dyke T et al. 2003. Pten dose dictates cancer progression in the prostate. *PLoS Biol* **1**(3): E59.
- Uziel T, Zindy F, Xie S, Lee Y, Forget A, Magdaleno S, Rehg JE, Calabrese C, Solecki D, Eberhart CG et al. 2005. The tumor suppressors Ink4c and p53 collaborate independently with Patched to suppress medulloblastoma formation. *Genes Dev* **19**(22): 2656-2667.
- Vernay B, Koch M, Vaccarino F, Briscoe J, Simeone A, Kageyama R, Ang SL. 2005. Otx2 regulates subtype specification and neurogenesis in the midbrain. *J Neurosci* **25**(19): 4856-4867.
- Visel A, Blow MJ, Li Z, Zhang T, Akiyama JA, Holt A, Plajzer-Frick I, Shoukry M, Wright C, Chen F et al. 2009. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* **457**(7231): 854-858.
- Wang Y, Yang J, Zheng H, Tomasek GJ, Zhang P, McKeever PE, Lee EY, Zhu Y. 2009. Expression of mutant p53 proteins implicates a lineage relationship between neural stem cells and malignant astrocytic glioma in a murine model. *Cancer Cell* **15**(6): 514-526.
- Wechsler-Reya RJ, Scott MP. 1999. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron* **22**(1): 103-114.

- Wortham M, Jin G, Sun JL, Bigner DD, He Y, Yan H. 2012. Aberrant Otx2 expression enhances migration and induces ectopic proliferation of hindbrain neuronal progenitor cells. *PLoS One* 7(4): e36211.
- Wortham M, Yan H. 2009. The use of retinoids as differentiation agents against medulloblastoma. In *CNS Cancer: Models, Markers, Prognostic Factors, Targets, and Therapeutic Approaches*, (ed. E Van Meir), pp. 1077-1104. Humana Press, New York.
- Xu W, Janss A, Packer RJ, Phillips P, Goldwein J, Moshang T, Jr. 2004. Endocrine outcome in children with medulloblastoma treated with 18 Gy of craniospinal radiation therapy. *Neuro Oncol* 6(2): 113-118.
- Yang ZJ, Ellis T, Markant SL, Read TA, Kessler JD, Bourbonoulas M, Schuller U, Machold R, Fishell G, Rowitch DH et al. 2008. Medulloblastoma can be initiated by deletion of Patched in lineage-restricted progenitors or stem cells. *Cancer Cell* 14(2): 135-145.
- Zakhary R, Keles, G.E., Aldape, K., and Berger, M.S. 2001. *Medulloblastoma and primitive neuroectodermal tumors*. Churchill Livingstone, London.
- Zhao H, Ayrault O, Zindy F, Kim JH, Roussel MF. 2008. Post-transcriptional down-regulation of Atoh1/Math1 by bone morphogenic proteins suppresses medulloblastoma development. *Genes Dev* 22(6): 722-727.
- Zheng H, Ying H, Yan H, Kimmelman AC, Hiller DJ, Chen AJ, Perry SR, Tonon G, Chu GC, Ding Z et al. 2008. p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. *Nature* 455(7216): 1129-1133.
- Zhu Y, Matsumoto T, Mikami S, Nagasawa T, Murakami F. 2009. SDF1/CXCR4 signalling regulates two distinct processes of precerebellar neuronal migration and its depletion leads to abnormal pontine nuclei formation. *Development* 136(11): 1919-1928.
- Zhuo L, Theis M, Alvarez-Maya I, Brenner M, Willecke K, Messing A. 2001. hGFAP-cre transgenic mice for manipulation of glial and neuronal function in vivo. *Genesis* 31(2): 85-94.
- Zindy F, Uziel T, Ayrault O, Calabrese C, Valentine M, Rehg JE, Gilbertson RJ, Sherr CJ, Roussel MF. 2007. Genetic alterations in mouse medulloblastomas and generation of tumors de novo from primary cerebellar granule neuron precursors. *Cancer Res* 67(6): 2676-2684.

Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* **393**(6685): 595-599.

## Biography

Matthew Jeremy Wortham was born in Columbia, MO on September 30, 1983. He graduated from Rock Bridge Senior High School in Columbia. He then attended William Jewell College, where he obtained a BA in Honors Molecular Biology from tutorial-based studies in the Oxbridge Program, which included a year of study at Cambridge University. Matthew developed an interest in biomedical research at William Jewell, which was further enhanced as he worked as a Research Assistant in the lab of Yu-Jui Yvonne Wan studying xenobiotic metabolism and fatty liver disease. He then joined the Cell and Molecular Biology umbrella program at Duke, which led him to join Hai Yan's lab in the department of Pathology. His experience in Hai's lab and his interactions with other Duke scientists fostered an interest in the role of developmental pathways in cancer, aging, and tissue repair. He has presented his work at the AACR Annual Meetings (2010 and 2012), the Pediatric Neuro-Oncology Basic & Translational Research Conference (2011), and the Pediatric Brain Tumor Foundation Institute Research Review Meeting (2012). He is an Associate Member of the American Association for Cancer Research (AACR), and was a member of the American Association for the Advancement of Science from 2007-2011. He received the AACR-Sanofi-Aventis Scholar-in-Training Award in support of his presentation at the AACR Annual Meeting in 2010. His publications are as follows:

- Guo C\*, Chang CC\*, **Wortham M**, Chen LH, Kernagis DN, Qin J, Cho YW, Chi JT, Grant GA, McLendon RE, Yan H, Ge K, Papadopolous N, Bigner DD, He Y. Global identification of MLL2-targeted loci reveals MLL2's role in diverse signaling pathways. *PNAS*. In Press. \*equal contribution.
- Shibata Y, Sheffield NC, Fedrigo O, Babbitt CC, **Wortham M**, Tewari AK, London D, Song L, Lee BK, Iyer VR, Parker SCJ, Margulies EH, Wray GA, Furey TS, Crawford GE. 2012. Extensive evolutionary changes in regulatory element activity during human origins are associated with altered gene expression and positive selection. *PLoS Genet* 8(6):e1002789.
- Wortham M**, Yan H. 2012. Apples to origins: Identifying brain tumor stem cell genes by comparing transcriptomes of normal and cancer stem cells. *Cancer Discov* 2(6):492-4.
- Wortham M**, Jin G, Sun JL, Bigner DD, He Y, Yan H. 2012. Aberrant Otx2 expression enhances migration and induces ectopic proliferation of hindbrain neuronal progenitor cells. *PLoS One* 7(4):e36211.
- Adamson DC\*, Shi Q\*, **Wortham M\***, Northcott PA, Di C, Duncan CG, Li J, McLendon RE, Bigner DD, Taylor MD, Yan H. 2010. OTX2 is critical for the maintenance and progression of Shh-independent medulloblastomas. *Cancer Res* 70(1):181-91. \*equal contribution.
- Wortham M**, Yan H. 2009. The use of retinoids as differentiation agents against medulloblastoma. In *CNS Cancer: Models, Markers, Prognostic Factors, Targets, and Therapeutic Approaches*, (ed. E Van Meir), pp. 1077-1104. Humana Press, New York.
- Wortham M**, He L, Gyamfi M, Copple BL, Wan YJ. 2008. The transition from fatty liver to NASH associates with SAME depletion in db/db mice fed a methionine choline-deficient diet. *Dig Dis Sci* 53(10):2761-74.

**Wortham M**, Czerwinski M, He L, Parkinson A, Wan YJ. 2007. Expression of constitutive androstane receptor, hepatic nuclear factor 4 alpha, and P450 oxidoreductase genes determines interindividual variability in basal expression and activity of a broad scope of xenobiotic metabolism genes in the human liver. *Drug Metab Dispos* **35**(9):1700-10.