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MECHANISMS AND TARGETING OF NEURODEVELOPMENTAL REGULATOR REST IN MEDULLOBLASTOMA DISSEMINATION

Keri Callegari

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MECHANISMS AND TARGETING OF NEURODEVELOPMENTAL REGULATOR REST IN MEDULLOBLASTOMA DISSEMINATION

by

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MECHANISMS AND TARGETING OF NEURODEVELOPMENTAL REGULATOR REST IN MEDULLOBLASTOMA DISSEMINATION

A

DISSERTATION

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for the Degree of

DOCTOR OF PHILOSOPHY

by

Keri Callegari, B.S.

Houston, Texas

May 2018
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Molecular subgrouping of medulloblastoma (MB) has produced four subgroups: wingless (WNT), sonic hedgehog (SHH), group 3, and group 4. While patients with WNT tumors have the best prognosis, patients with SHH tumors have a more variable prognosis concurrent with metastatic disease. This subset of SHH patients have elevated levels of the neurogenic regulator, RE1 Silencing Transcription factor (REST). To understand the role of REST in MB, we utilized a novel transgenic mouse model wherein REST expression can be conditionally elevated during postnatal development in the cells of origin of SHH MB, cerebellar granule neural progenitors (GNPs). While these mice did not form tumors, an abnormal organization of GNPs in the cerebella of these mice was observed. Molecular investigation of these REST-high GNPs revealed heightened expression of the chemokine receptor, C-X-C-motif receptor 4 (CXCR4). CXCR4 function has established importance in both cerebellar neurodevelopment and MB tumor metastasis. In MB mouse models, increased REST expression resulted in increased CXCR4 expression that was related to increased tumor dissemination and dramatically reduced survival of mice. Analysis of patient samples revealed that REST and CXCR4 levels were concurrently high in patients with metastasis in a subset of SHH (SHH-beta) patients. Examination of human MB cell lines strengthened the observation that REST elevation increased MB cell...
migration. The final section of my dissertation work has focused on targeting REST activity through inhibition of epigenetic cofactor, lysine specific demethylase 1 (LSD1). To this end, I examined human MB patient samples for LSD1 expression and discovered that while CXCR4 signaling was highly relevant to SHH-beta subgroups, LSD1 was increased in the context of REST most strongly in SHH-alpha and SHH-beta patient groups. *In vitro* work demonstrated that LSD1 knockdown reduced SHH MB cell line viability in the context of normal and high REST. However, irreversible inhibition of LSD1 had no effect on cell viability but did exert a REST-dependent reduction of migration. Ingenuity pathway analysis of RNA-sequencing data revealed that irreversible inhibition of LSD1 reduced cell migration signaling pathways relevant to MB. In summary, my work has two major findings. First, in the context of REST elevation, CXCR4 expression and signaling increases along with migratory capacity of postnatal GNPs that contribute to perturbed development and medulloblastoma infiltration. Second, REST activity can be targeted against complex member LSD1 and pharmacological targeting of LSD1 with irreversible inhibitors blocks REST-dependent MB cell migration. Collectively, my work has demonstrated that elevated REST expression in the developing brain can perturb neurodevelopment and influences migration signaling pathways to contribute to medulloblastoma dissemination.
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Abbreviations

µm – Micrometer

µM – Micromolar

AKT – Protein kinase b

APC – adenomatous polyposis coli

BBB – Blood brain barrier

BLI – Bioluminescence imaging

B-TRCP - Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase

CALM - Calmodulin

CK1α – Casein kinase 1 alpha

CNS – Central Nervous System

CoREST – REST corepressor

CSF - Cerebral Spinal Fluid

Ct – Cycle threshold

CXCR4 – Chemokine C-X-C receptor 4

DMEM – Dulbecco’s Modified Eagle Medium

DNA – Deoxyribonucleic acid

DVL – Dishevelled

EGL – External granule cell layer
FACS – Fluorescence-associated Cell Sorting

FACS – Fluorescence-associated cell sorting

FBS – Fetal Bovine Serum

FFLUC – Firefly luciferase

FGF8 – Fibroblast growth factor 8

G9a – Euchromatic histone-lysine N-methyltransferase 2

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GBX2 – Gastrulation brain homeobox 2

GFAP – Glial fibrillary acidic protein

GFP – Green fluorescent protein

Gli – Glioma associated transcription factor

GNP – Granule neural progenitor

GRK5/6 – G protein related kinase 5/6

GSK3β – glycogen synthase kinase 3 beta

H&E – Hematoxylin and eosin

H3 – Histone 3

H3K4 – Histone 3 lysine 4

H3K9 – Histone 3 lysine 9

HA - Hemagglutinin
HBP – Hedgehog binding protein

HDAC – Histone deacetylase

IACUC – Institutional Animal Care and Usage Committee

IGL – Internal granule cell layer

IHC - Immunohistochemistry

IVIS – In vivo imaging system

kDa – Kilodalton

LRP – low density lipoprotein receptor related protein

LSD1 – Lysine specific demethylase 1

MAGIC – Medulloblastoma Advanced Genomic International Consortium

Math1 – Proneural gene atonal homolog 1

MB - Medulloblastoma

MDACC – MD Anderson Cancer Center

MEM – Modified Eagle Medium

ML – Molecular layer

MRI – Magnetic Resonance Imaging

NeuN - Hexaribonucleotide Binding Protein-3

NIH – National Institute of Health

NSG – NOD-scid gamma
OTX2 – Orthodenticle homeobox 2

PBS – Phosphate buffered saline

PBS-T – Phosphate buffered saline - tween

PDOX – Patient-derived orthotopic xenograft

PKa – Protein kinase a

PL – Purkinje cell layer

PNET – Primitive Neuroectodermal Tumor

PTCH1 – Patched homologue 1

Ptf1a – Pancreas transcription factor 1 a

qRT-PCR – Real-time quantitative reverse-transcriptase polymerase chain reaction

RE-1 – Restricted element 1

REST – Restricted Element 1 Silencing Transcription Factor

REST^TG – REST-transgenic

RL – Rhombic lip

RNA – Ribonucleic acid

rpm - rotations per minute

RTPS – Rhabdoid tumor predisposition syndrome

SDF1/CXCL12 – Stromal Derived Factor 1/ Chemokine C-X-C motif ligand 12

SHH – Sonic Hedgehog
**SKP1** - S-phase kinase-associated protein 1

**SMO** – Smoothened receptor

**β-cat** – beta-catenin

**Sufu** – Suppressor of fused homolog

**TBS** – Tris buffered saline

**TBS-T** – Tris buffered saline – tween

**TCF/LEF** – T-cell factor/lymphoid enhancing factor

**TUBB3** – Class III beta-tubulin

**VN** – Ventricular neuroepithelium

**WHO** – World Health Organization

**WNT** – Wingless

**WT** – Wild-Type
Chapter 1: Introduction
1.1 Medulloblastoma and pediatric brain cancer

1.1.1 Epidemiology and clinical features

Cancer is the second most common cause of death in children under 14 years of age. While relatively rare in adults, central nervous system (CNS) tumors account for 1 in 4 childhood cancers and are second only behind childhood leukemia (Figure 1). While childhood leukemia has a higher incidence, the leading cause of cancer death is CNS tumors (1). As of 2017, overall ratios comparing sex differences in CNS tumor incidence and mortality reveal a slight increase in susceptibility of CNS tumors in male patients, with a 1.4 male to female incidence ratio and a 1.5 male to female mortality ratio (1).

Tumor grade classifications of CNS tumors are based on pathological appearance of the tumor tissue. Tumor grades range from I to IV and are established indicators of how rapidly a tumor is likely to grow and spread. After development, normal cells of the brain differentiate into specialized cells that do not migrate or multiply often. Low-grade tumors contain mostly cells that are differentiated while high-grade tumors consist of undifferentiated cells that are rapidly dividing and poorly organized. Across CNS tumors, astrocytomas are the most common tumor type. However, a portion of these tumors are low grade and not malignant. The most common high-grade, malignant pediatric CNS tumor is medulloblastoma (MB), making up 20% of all tumors (2). 70% of MB patients are under the age of 20 and MB tumor cells are of embryonic origin.
Figure 1. Cancer incidence in patients aged 0-14 years. Together, acute lymphoblastic leukemia (26%) and acute myeloid leukemia (5%) make up the most common group of childhood cancers. At a 21% incidence, CNS tumors measure up as the second most common childhood cancer. Childhood cancer is the second leading cause of death, only behind accidents in children from the United States. Data acquired from American Cancer Society “Cancer Facts & Figures 2014 (includes the Special Section: Childhood and Adolescent Cancer)”.
MB is a world health organization (WHO) grade IV, primitive neuroectodermal tumor (PNET) that arises subtentorially in the posterior fossa of the brain. The posterior fossa includes the hindbrain which contains the cerebellum, medulla, and pons. The cerebellum is the most frequent lesion location for MB and is a highly conserved structure important for balance and movement. Due to this, tumor growth here often produces symptoms like abnormal gait, lack of coordination, and ataxia. Another side effect of growing tumors may be increased intracranial pressure due to blockages in the ventricular system (Figure 2). Throughout the tissue of the brain, cavities called ventricles are filled with cerebral spinal fluid (CSF). CSF is produced by cells within the choroid plexus and circulates throughout the CNS within the ventricular space. When tumors grow they may occlude the ventricular space. This occlusion can create a build-up of CSF in the brain resulting in increased intracranial pressure that contributes to symptoms such as headache, nausea, vomiting, and behavioral changes. Pressure on cranial nerves due to tumor growth may also cause patients to present with specific ocular and oculomotor dysfunctions, such as double vision, dizziness, and/or an inability to focus or follow moving visual stimuli.

Clinical diagnosis of MB is accomplished by performing a magnetic resonance imaging (MRI) scan of the patient’s brain with gadolinium contrast-enhancement. These MRI agents are able to enter the brains of MB patients due to tumor-associated degradation of the blood brain barrier (BBB). Gadolinium-containing agents will permeate tumors with increased vascularity and increase contrast in tumors resulting in a defined tumor image. If the tumor is a large defined mass in the cerebellum with uniform contrast-enhancement (although many MB tumors also have necrotic areas that can leave a more
variable pattern), the diagnosis will likely be MB. As 30% of MB tumors spread to other CNS locations via CSF, MRI imaging is also performed throughout the spinal cord to check for metastasis (3, 4). Another method to check for potential metastasis is to collect CSF via lumbar puncture and cytologically analyze the sample to validate whether free-floating tumor cells are traveling in the CSF. Confirmation of MB diagnosis is achieved by histopathology of a biopsy taken at surgical resection. MB has four histological variants: Classic, Desmoplastic/Nodular, Desmoplastic/Extensive Nodularity, and Large Cell/Anaplastic. Classic MB presents with numerous, densely-packed cells with small round nuclei. Desmoplastic MB is also highly cellular but contains “nodes” of densely packed cells intermixed with more loosely-packed areas of low cellularity. Large cell/anaplastic MB contain large abnormally shaped cells with high proliferative capacity.

Once MB diagnosis is confirmed, clinical risk can stratify patients into two groups: average-risk and high-risk. Patients are considered high-risk if they are less than 3 years of age, if there is evidence of disseminated disease, and/or if the tumor resection was subtotal (leaving >1.5 cm² residual tumor on post-operative MRI) (5, 6). Other high-risk factors include large cell/anaplastic tumor histology and molecular expression profiling indicative of drug resistance or tumor aggression. Patients that are not high-risk are considered average-risk and receive the standard of care which includes local radiation therapy and cytotoxic chemotherapeutic drugs. High-risk patients have their spinal cords irradiated in addition to focal radiation. Patient prognosis and prediction to standard treatment was historically based on clinical risk stratifications and histological analyses. However, these clinical means alone were not sufficient to truly predict patient outcomes.
Figure 2. Overview of human brain structure and meningeal layering. Sagittal brain section showing the cerebral spinal fluid (CSF) filled ventricular system (blue). The meninges contain three distinct layers: dura mater, arachnoid mater, and pia mater. CSF flows through the subarachnoid space to encapsulate the brain and the ventricles contain the choroid plexus which produce CSF. Tumors in the brain stem or cerebellum are subtentorial while tumors in the cerebrum are supratentorial. Subtentorial tumors may grow large enough to block the fourth ventricle to increase intracranial pressure.
1.1.2 Molecular mechanisms and subgrouping

In attempts to better understand the disease and anticipate patient prognosis, various classifications of MB have been established in the past decade. Previous classifications of MB were largely determined by clinical manifestations and histology which limited predictive value of patient prognosis and treatment aggression. Thanks to the medulloblastoma advanced genomic international consortium (MAGIC) and sequencing technology advances in the last decade, MB tumors are now further stratified based on their molecular make-up. Molecular-based classification of MB has proven to predict patient outcomes more accurately than previous methods alone. This movement has also paved the way for research into targeted therapies, an area in which pediatric brain cancers are severely lacking. In this project, MAGIC analyzed mRNA and miRNA expression profiles of 1000 human MB tumor samples.

Currently, molecular classification of MB contains 4 subgroups: Wingless (WNT), Sonic Hedgehog (SHH), Group 3, and Group 4 (7, 8). These subgroups attain their namesake through characteristic activation of developmental molecular signaling pathways, excluding the less well understood groups 3 and 4. WNT MB tumors are characterized by over-activated WNT signaling and have a mostly classic histology. Patients with WNT tumors have very good prognosis and can usually be spared from a harsh treatment regimen. WNT patients have a long-term survival probability of 90% and tumors rarely metastasize or recur after surgery. SHH MB tumors are characterized by either perturbed SHH signaling that drives tumor initiation. While most desmoplastic tumors are SHH, only 50% of SHH tumors are desmoplastic with the other remaining half being either classic or large cell/anaplastic. SHH MB has an interesting temporal
incidence as it is frequent in both infants (0-3) and young adults (16+) while relatively rare in patients between the ages of 3 and 16. Patient prognosis is mixed with subset of patients experiencing very aggressive disease and metastasis. Group 3 MB tumors are considered the most aggressive of the four classifications and frequently metastasize and recur. Histology of Group 3 tumors are usually classic or large cell/anaplastic and many of these tumors are characterized by MYC oncogene amplification. The MYC gene encodes a transcription factor responsible for cell cycle progression, apoptosis, and cell transformation. Patient prognosis has been shown to vary with MYC amplification, as patients with MYC overexpression have a higher rate of recurrence and death. Group 4 MB tumors constitute over 30% of MB diagnoses but are the least well understood MB subgroup. Patient prognosis is intermediate and interestingly, while males have a higher incidence of Group 4 MB (2:1; male:female), 80% of females with Group 4 MB exhibit loss of an X chromosome. Gene expression profiling of Group 4 tumors also demonstrate an overexpression of neuronal differentiation/development genes. In this past year, collaborators of the MAGIC project have further subclassified these four molecular subgroups into up to four subtypes (α, β, δ, γ; Figure 3) (7).

Molecular classification of MB tumors has allowed for much progress in illuminating new molecular targets for potential therapeutic use in MB. A major unmet clinical need is the identification of targets for treatment of metastatic disease and the drivers of these tumor behaviors are largely unknown. My thesis work described here has examined the molecular basis of MB dissemination, with a specific focus on SHH MBs, where a subset of patients (α and β) exhibit metastasis. The cells of origin of SHH MB are known and mouse models are available making molecular research possible.
Figure 3. Graphical summary of 12 medulloblastoma subtypes. WNT, SHH, Group 3, and Group 4 MB tumors are further subtyped into up to four subtypes (α, β, δ, γ). Age groups are defined as infant (0-3), child (3-10), adolescent (10-17), and adult (>17). Figure reprinted with permission from Cell Press (Cavalli, et al. 2017. Intertumoral Heterogeneity within Medulloblastoma Subgroups. Cancer Cell 31: 737-754 e736.).
1.2 At the intersection of normal development and medulloblastoma

1.2.1 Prenatal development in brief

The process of mammalian development begins when two gametes (sperm and egg) fuse to mark fertilization. Each gamete combines into a single-cell zygote, which contains all of the genetic information required to generate a fully developed organism. The zygote undergoes four initial, symmetrical divisions to initiate the developmental process known as cleavage to result in a 16-cell morula. At this point, blastulation begins and the cells divide asymmetrically (Figure 4). To give rise to the blastocyst, cells differentiate into an outer cell layer (trophoblasts) and an inner cell mass (embryoblasts). As the embryoblasts differentiate, they polarize within a ring of the trophoblasts leaving an empty cavity termed the blastocoele. The embryoblast will ultimately give rise to the embryo and amnion while the trophoblasts become the fetal part of the placenta. The blastocyst implants into the uterine wall to receive oxygen and nutrients from the mother and complete conception. The next step of embryogenesis is gastrulation, whereby the blastula reorganizes into a multilayered structure known as a gastrula. During gastrulation, the anterior to posterior (head to tail) orientation, ventral to dorsal (front to back) orientation, and the three germinal layers are established. The germinal layers are formed as three disks (in order from deepest to most superficial): the endoderm, mesoderm, and ectoderm. The endoderm will develop into many interior organs such as the intestines, lungs, pancreas, thyroid, and bladder. The mesoderm will spawn the heart, bones, muscles, and the beginnings of the circulatory system. And finally, the ectoderm layer gives rise to many connective tissues, the eyes, inner ear, outermost layer of skin, and central and peripheral nervous systems.
Figure 4. Graphical representation of blastulation and neurulation. (top) Fertilization results in a zygote that undergoes 4 symmetrical divisions to become a morula. Cells within the morula differentiate to allow the formation of a blastocyst. (bottom) Following gastrulation and the generation of the three germinal layers (ectoderm, mesoderm, and endoderm), the layers organize into the developing embryo. A section of the ectoderm, the neural plate, folds inwards on itself to form the neural tube which runs the length of the embryo.
Focusing on the nervous system from here on, human brain development begins as early as the third week of gestation and continues until full brain maturation in adulthood (Figure 4). A specialized area of the ectoderm, the neuroectoderm, gives rise to the neural plate. The neural plate is the origin of the entire nervous system and folds in on itself to form a tube with open ends. By the fourth week of gastrulation, the open ends seal and create a closed neural tube. At this point, the neural tube can be divided into 3 major subdivisions that give rise to the different parts of the CNS (Figure 5). These divisions begin at the anterior end with the prosencephalon (contains the telencephalon (cerebrum) and the diencephalon (the optic vesicles and hypothalamus)). The middle region is the mesencephalon (midbrain) and the posterior end is the rhombencephalon (contains the metencephalon (the pons and cerebellum) and the myelencephalon (the medulla oblongata)). The spinal cord arises from the notochord. Further stratification of these areas will give rise to specialized regions in the brain.

The metencephalon undergoes a few molecular signaling cascades to remain separate from the mesencephalon and to begin development of the hindbrain structures of the pons and cerebellum. Tight regulation of secreted factor fibroblast growth factor 8 (FGF8) establishes and maintains this barrier (9). Secreted at the barrier between the two vesicles, FGF8 acts as an inhibitor of orthodenticle homeobox 2 (OTX2) expression and is coexpressed with gastrulation brain homeobox (GBX2). OTX2 expression helps establish the mesencephalic territory while GBX2 expression is responsible for the metencephalic region. Temporospatal expression of these players, among others, are critical to proper cerebellar patterning and development.
Figure 5. Primary and secondary vesicle stages of development. After neural tube formation, 3 vesicles are formed with the Prosencephalon being most anterior. Secondary vesicle development further divides these regions into 5 developmental regions.
The cells that populate the mature cerebellum arise from two germinal zones known as the ventricular neuroepithelium (VN) of the fourth ventricle and the rhombic lip (RL) \(10\text{-}12\). The VN produces pancreas transcription factor 1 a (Ptf1a+) positive GABAergic neurons, including Purkinje cells and postnatal interneurons, and a majority of the glia \(13\). The rhombic lip is the origin of proneural gene atonal homolog 1 positive (Math1+) glutamatergic cells and has two distinct upper and lower areas. The upper rhombic lip mainly exists as a source of granule neural progenitors (GNPs) while the lower rhombic lip largely gives rise to several precerebellar nuclei \(14\text{-}16\). GNPs produced by the upper rhombic lip make up the majority of the neuronal cell population in the cerebellum and are the most abundant neuron in the brain \(17\).

To populate the prenatal cerebellum, Math1+ GNPs migrate rostrally from the RL and disperse across the dorsal side of the cerebellar anlage. As these progenitors fill out the outermost area of the cerebellar structure, they begin to define what is known as the external granule cell layer (EGL). Purkinje cells from the VN migrate micrometers below the EGL in the Purkinje cell layer (PL). The space in between these two layers is the molecular layer (ML). Further inwards from the PL, is the internal granule cell layer (IGL). At this point in development, before birth, the IGL is populated by interneurons and glia (the projections of which extend into the EGL). However, in the fully developed cerebellum, the IGL mostly contains mature granule neurons.
1.2.2 Postnatal development in brief

Cerebellar development continues to occur postnatally (Figure 6). Predominantly residing in the EGL, GNPs proliferate in response to a number of stimuli including SHH secreted from the Purkinje cells. At approximately postnatal day 5-8 in mice, the GNPs are at the peak of their proliferation. GNPs in the outer EGL proliferate every 18-20 hours and this proliferative phase lasts up to 3 weeks in mice and 2 years in humans (18, 19). As cells begin the differentiation process, they become post-mitotic and enter the inner EGL. Within the inner EGL, GNPs extend small horizontal processes and initiate migration tangentially across the EGL over the span of 24-48 hours. Upon contact of the GNP with a projection from a highly-specialized radial glia, Bergmann glia, radial migration is initiated. GNPs take about 10-11 hours to traverse the molecular layer and reach the Purkinje cell layer in this glia-associated migration. At this point, the cells detach from the glial process and begin glia-independent migration through the IGL until a leading process reaches white matter (18, 20, 101).

The molecular mechanisms of postnatal GNP migration in the cerebellum are complex and continue to be investigated. Molecules involved in tangential migration include neurotrophin-3, stromal-derived factor 1 (SDF1), ephrin-B2, and others (21-23). Downstream signaling pathways activated by these molecules include calcium signaling, cyclic nucleotide signaling, JAK/STAT signaling, and mitogen-activated protein kinase (MAPK) signaling. Mechanistically, tangential migration is spurred by secretion of a potent chemoattractant, SDF1, by meningeal cells encapsulating the EGL. SDF1 binds to the chemokine C-X-C motif receptor 4 (CXCR4) available on the cell surface of GNPs. Tangential migration is postulated to help retain cells in the EGL and prevent its premature
depletion. Tangential migration ceases when the GNP is in proximity of a projection from. Due to still disputed mechanisms, the GNP switches off its tangential migration machinery and begins glial-associated radial migration into the IGL. This radial migration is established to be dependent on a multitude of signaling stimuli including ephrin-B2 signaling, brain derived neurotrophic factor (BDNF) secretion, secreted glial factors (astrotactin, tenascin, neuregulin, and fibronectin) \((101)\). Done successfully, postnatal GNP proliferation and migration results in a complex laminated cerebellar structure capable of encoding and transmitting complex neural data.
Figure 6. Postnatal cerebellar lamination. Proliferating GNPs respond to SHH secreted by the Purkinje cells. As they begin the differentiation process, they migrate tangentially across the EGL before finding a Bergmann glia projection and migrating radially inwards. The mature granule neuron will reside in the IGL for the remainder of its lifespan.
1.2.3 Developmental signaling in cancer

The quest to elucidate mechanisms of MB etiology has discovered various familial developmental disorders related to tumorigenesis. Hereditary syndromes that alter developmental signaling and predispose children to MB development include Li-Fraumeni syndrome, Turcot syndrome, Gorlin’s syndrome, and rhabdoid tumor predisposition syndrome (RTPS). Patients with these autosomal dominant disorders have a higher incidence of CNS tumor development, including MB (24, 25).

Li-Fraumeni syndrome patients are plagued by a wide-range of cancers arising from germline mutations of the apoptotic and cell cycle regulator, P53 (26, 27). These loss of function mutations cause reduced checkpoint control, resulting in unfettered proliferation and hindered apoptosis contributing to tumorigenesis in various cell types throughout the patient’s body. Turcot syndrome type 2 patients carry a germline mutation of the adenomatous polyposis coli (APC) gene which regulates the degradation of beta-catenin, a WNT pathway player. Loss of function of APC and subsequent accumulation of beta-catenin permits overactive WNT signaling and promotes polyposis and lipoma, skin cell carcinoma, and brain tumor formation (Figure 7) (28-30). In mouse models, loss of function mutations in APC or gain of function mutations in beta-catenin in combination with P53 mutations in brain lipid binding protein (BLBP) positive cells of the lower RL give rise to WNT subgroup MB. Examination of Turcot/Li Fraumeni patients and lineage tracing experiments identified BLBP+ progenitors of the lower RL as cells of origin of WNT MB (31, 32).

Gorlin’s syndrome patients are hallmarked by tumor formation, macrocephaly, and skeletal and facial abnormalities (33). Tumorigenesis in these patients is caused by a loss
of function mutation in the SHH pathway regulator, patched homologue 1 (PTCH1) (34). PTCH1 is a twelve transmembrane domain containing receptor that negatively regulates smoothened (SMO) localization and signaling when not bound by the SHH ligand (Figure 8). SMO is a seven-transmembrane domain containing receptor that initiates a signaling cascade to activate glioma-associated (Gli) transcription factors 1 and 2. Normally, in the absence of SMO activation, Gli is tightly regulated by several proteins. Gli can be sequestered by suppressor of fused homolog (Sufu) and/or phosphorylated by glycogen synthase kinase-3 beta (GSK3β), (CK1a), or site-specific phosphorylation of Gli by these proteins will either allow for ubiquination and subsequent degradation of Gli or proteolytic processing of Gli 1/2 into a Gli 3 isoform. While Gli 1 and 2 are transcriptional activators, Gli 3 exists in a NH₂-terminal truncated form and is thought to act as a transcriptional repressor (35). SHH binding to PTCH1 alleviates suppression of SMO and allows Gli 1/2 mediated transcription of SHH target genes. The amount of SHH available to bind to PTCH1 is tightly regulated by hedgehog-binding proteins (HBPs) which sequester SHH away from the PTCH receptor (36). Loss of function mutations of PTCH allow SHH signaling to go unchecked and contributes to MB development in GNPs of the cerebellum. The results of these discoveries permitted the generation of SHH MB mouse models with gain of function SMO mutations and loss of function PTCH1 mutations in GNPs (32, 37).

It is clear from a majority of the mechanistic studies that MB is a disease of hindbrain development gone awry (7, 8, 25, 28, 31, 32, 37). As MB is an embryonal tumor, there are many pathways that can be affected as described in this section and in the sections above. Postnatally, it is easy to postulate that spontaneous mutations or unregulated signaling in the cerebellum could be responsible for MB development. Due
to the spatiotemporal requirement of coordinated molecular signaling and cellular dynamics, the developing postnatal cerebellum lends itself vulnerable to insult and malformation. As previously mentioned, GNPs are established as the cells of origin of SHH MB. Developmental signaling pathways involved in GNP renewal and maturation have already been implicated in MB pathogenesis. Further, “traffic jams” due to improper migration of GNPs of these cells can also contribute to MB initiation and progression (38-41). For example, overexpression of CXCR4 and SDF1 is observed in MB patient tumor samples and the levels of these proteins is negatively correlated with predicted patient prognosis (8, 42, 43). Some subsets of MB, like SHH MB, have been extensively studied for mechanisms of their CXCR4 overexpression and expression of CXCR4 in these tumors has been shown to be largely dependent on SHH activity. The dysregulation of CXCR4 has also been demonstrated to occur both within and outside of the context of P53 (39, 44, 45).

As described in the next section, another molecule of increasing interest is the canonical neurogenic regulator, restricted element 1 (RE-1) silencing transcription factor (REST). Expression of REST has been linked to worsened patient prognosis and leptomeningeal dissemination of MB (46, 47). Unpublished work from our lab shows that the mechanisms of REST as driver of MB progression is partially due to REST repression of PTCH1 and activation of AKT signaling (Dobson 2018 – under review). However, the mechanisms by which REST promotes MB tumor dissemination are not clear and is the major question of my thesis project.
Figure 7. Graphical summary of canonical WNT signaling pathway. When the transmembrane receptor Frizzled is unbound by WNT ligand, it binds and inactivates Dishevelled (Dvl). In the cytoplasm, beta-catenin (β-cat) is bound by a complex comprised of axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3 beta (GSK3β). This complex site-specifically phosphorylates β-cat and directs it to be ubiquinated by beta-transducin repeat containing E3 ubiquitin protein ligase (β-trcp). In its activation state, when WNT is bound to Frizzled, low density lipoprotein receptor related protein 5/6 interacts with Frizzled and axin while Dvl is released. Dvl is free to disrupt the axin/APC/GSK3β complex and permits the accumulation of β-cat in the cytoplasm. In this state, β-cat can translocate to the nucleus and switch on gene expression alongside T-cell factor/lymphoid enhancing factor (TCF/LEF).
**Figure 8. Graphical summary of SHH signaling.** In the inactive state, the patched receptor (PTCH1) indirectly inhibits membrane localization of the smoothened receptor (SMO). Gli transcription factors 1 and 2 are site-specifically phosphorylated by protein kinase a (PKa), GSK3β, and casein kinase 1 alpha (CK1α). This phosphorylation either leads to truncation of the NH₂-terminus to produce the repressive Gli 3 isoform or ubiquitination by β-trcp. Suppressor of fused homolog (Sufu) also binds Gli 1/2 and sequesters it away from functional interactions in the cytoplasm and nucleus. As a ligand, SHH can be sequestered away from PTCH1 by hedgehog binding proteins (HBPs) in the extracellular space. In the cases above, SHH target gene transcription is repressed. When SHH binds to PTCH1, its repression on SMO is ameliorated. Through mechanisms not yet fully understood, phosphorylation of Gli by PKa, GSK3β, and CK1α do not occur and Gli 1/2 is able to translocate to the nucleus and turn on SHH target gene expression.
1.3 RE-1 silencing transcription factor (REST) in tumor progression

1.3.1 The field of epigenetics

The field of epigenetics (and the term itself) was born from experiments by Conrad Hal Waddington in 1956. He demonstrated that the thorax and wings of embryo fruit flies (Drosophila) could be influenced to develop in different ways based on the exposure of the Drosophila embryo to a wide range of temperatures or chemical stimuli \(^{(48)}\). While the underlying genetic code was the same in each embryo, environmental stimuli could alter the fate of developmental process. To further strengthen these experiments, Waddington continued to breed these flies and found that even without continued exposure to the stimuli and within a few generations, these phenotypes persisted in the resulting offspring. This process was termed genetic assimilation, whereby these genetic decisions became locked in and a permanently heritable trait.

The results from his experiments led him to illustrate a model that could postulate how the same pluripotent, embryonic cells could differentiate into distinctly different cell types. He would represent this developmental landscape as a marble on top of a grooved hill where a series of decisions would favor one route over another and ultimately lead to one fate versus another \((\text{Figure } 9)\). However, the question still left open by these studies was whether or not the genetic information was lost with these decisions. Within the decade, another set of experiments found that the nucleus of an adult differentiated cell still contained all of the genetic information as that from an embryonic one \(^{(49)}\). This was accomplished by transferring the nucleus from a somatic cell in an adult frog to an unfertilized egg. The results demonstrated that the synthetically fertilized egg could fully develop without complication.
Figure 9. The developmental landscape. The marble represents a pluripotent cell with each decision being a molecular event that restricts the potential of cell to become anything else until, finally, the cell reaches its ultimate and specialized cell fate. It is speculated on whether this model is relevant in stem cell reprogramming. If cells are pushed earlier into the undifferentiated form, can the marble truly move back up from their specialized groove to become multipotent at one of the transitional grooves? Or are there mechanisms that do not allow for full reprogramming?
The results of these two monumental experiments primed the postulation that there must be a mechanism to modify and regulate the expression of genes without removing or losing genetic material. However the discovery that all of the genetic material remains intact in every cell, gave rise to two new questions. How does all of the genetic material fit into one cell and how is expression of the exact same genetic material regulated to give rise to different cell types?

Our genetic code is made up of double-stranded deoxyribonucleic acid (DNA). DNA is built with a series of nucleotides that code for instructions to transcribe ribonucleic acid (RNA) that is translated into functional protein units. One human cell contains approximately 2 meters DNA in a nucleus with only a 6 micrometer (μm) diameter. Further, thanks to its negatively charged phosphate groups, DNA is a highly negative polymer that needs to be packaged in a way that is accessible to transcriptional machinery and will also not break or tangle. The solution to this packaging conundrum is a series of folding and condensation steps into what we know as chromosomes.

The normal human cell contains 24 chromosomes. Each chromosomes contains one very long DNA molecule that is folded with proteins to form a compact structure called chromatin. Chromatin is made up of DNA that is folded around positively-charged proteins known as histones. Histone proteins exists as octamers that act as spools which DNA wraps around 2.5 times before extending “linker DNA” to the next histone. The chromatin structure containing these histone spools, and ~146 base pairs of wrapped DNA, occurs as a “beads on a string” and the units of these beads are termed nucleosomes.

Nucleosomes are the basic unit of the chromosome structure. The octamer of histones making up the “bead” of the chromosome contain two copies of histone H2A,
H2B, H3, and H4 proteins. These histones have “tails” of modifiable amino acids. Arginine (R) and lysine (K) residues are popularly modified to influence both chromatin structure and recruitment of transcription factors (50-52). These histone tail residues are available in the nuclear space to be read and modified by epigenetic enzymes known as readers and writers, respectively.

Some histone tail modifications modify the charge of the protein, either compacting the chromatin further (which impedes transcription) or loosening the chromatin to allow improved access of genes to transcriptional machinery. For example, histone acetylation (the addition of an acetyl group to a histone tail residue) classically neutralizes the histones positive charge to contribute to a more open chromatin structure and render the DNA more accessible (53). On the other hand, the addition of a methyl group to residues (methylation) will hinder the acetylation of the residue and can alter the association of other transcription factors. While methylation of H3K9 and H3K27 residues lead to repressive states gene expression. Methylation of H3K4 can actually promote transcription factor association with the region. Other modifications will promote or deter the association of other enzymes including nucleosome remodelers and transcriptional machinery. It is also important to note that DNA itself can be methylated and methylation occurring on cytosine-phosphate-guanine (CpG) islands found near the transcription start site (TSS) of genes silences expression.
**Figure 10. Chromosomal structure.** DNA is wrapped around histone protein octamers that comprise nucleosomes. Nucleosomes are arranged in a “bead on the string” fashion that is dynamic and can be found in an open or closed conformation that directly affects gene expression. Chromatin fibers are made of 30 nanometers of packed chromatin that can be further condensed into coils. These coils condense into supercoils that make up the hallmark chromosomal shape. *Image adapted and reprinted with permission from Molecular Cell Biology, 2007 textbook section C “Cell Division, Genetics, and Molecular Biology” (Lodish H, Berk A, Zipursky SL, et al. Molecular Cell Biology. 6th edition. New York: W. H. Freeman; 2000).*
1.3.2 Canonical REST activity as a chromatin remodeler

REST is a 121 kilodalton (kDa) kruppel-type zinc finger transcription factor. The canonical function of REST is to repress neuronal genes in non-neuronal cells and stem cells. REST is expressed ubiquitously throughout the body except within the CNS, where it is downregulated in differentiated neurons (54). As a master regulator of neurogenesis, REST represses neuronal gene expression by binding to 21-23 base pair sequence in the regulatory regions (known as RE-1 binding sites) of neuron-specific genes (54-56); Chong 1995). First discovered in 1995, REST has been implicated in several important diseases and functions including neurogenesis, neurodegenerative disease, cancer, ischemia, and aging (57-62).

While REST binds directly to DNA, REST itself is not responsible for changes in gene expression. Instead, REST associates with corepressor and chromatin remodeling enzymes through domains located at the amino (N)- and carboxy (C)- termini. Through its N-terminus, REST associates with the paired amphipathic helix protein Sin3a (mSin3A) corepressor complex. mSin3a further associates with histone deacetylases 1 and 2 which deacetylate amino acid residues on histone tails to curb transcriptional machinery interactions (63). Through its C-terminus, REST associates with the REST corepressor (CoREST) complex which is comprised of histone deacetylase 1/2 (HDAC1/2), lysine specific demethylase 1 (LSD1), euchromatic histone-lysine N-methyltransferase 2 (G9a), and others (64-66). These epigenetic players act on histone tail modifications that exist to alter the promotion of transcriptional machinery interaction with specific genes in a spatiotemporal manner.
1.3.3 REST in pediatric brain cancer

A potential role for REST as an oncogene was first described in a study of MB (67). REST expression was maintained across three MB cell lines and adenoviral recombinant activation of REST target gene expression decreased cell line viability partially though triggered apoptosis (67). In another study, REST expression was detectable in 17 out of 21 MB tumors (6 strong; 11 weak) (68). Further, adenoviral recombinant activation of REST target gene expression blocked tumor establishment in vivo. Interestingly, REST expression alone is not enough to induce tumorigenesis (69, 70). As for many oncogenes, including MYC, a second hit is required for tumorigenesis to occur. Mouse models expressing REST with MYC and REST with a lost allele of the PTCH1 receptor develop tumors over time (32, 37).

Experiments performed by previous members of the lab with mice expressing a human REST transgene in the context of cells with a lost PTCH1 allele (REST\textsuperscript{TG}/Ptch\textsuperscript{+/−}), demonstrated a dramatic increase in tumor burden coincident with a reduction in tumor latency and overall survival. Further examination of tumor samples from these mice revealed very aggressive tumor course and leptomeningeal dissemination. Mechanistically, these effects were attributed in part to functional loss of heterozygosity of the PTCH1 allele, leading to a homozygous null-like phenotype of SHH hyperactivity. Further, deregulation of downstream Gli proteins were also observed along with protein kinase b (AKT) signaling pathway hyperactivity. These results suggested a deregulation of proliferation, differentiation, and survival pathways converging to promote tumorigenesis. However, the mechanisms of how REST promotes dissemination remained to be understood.
In human samples, elevated REST levels are negatively correlated with patient prognosis (47, 67, 71). Increased gene expression and protein levels of REST are correlated with poor overall and event-free survival. Further, inhibition or loss-of-function mutations in the ubiquitin ligase complex responsible for degrading REST protein have been described and implicated in MB. The SCF (SKP1-CUL1-F-box protein) ubiquitin ligase complex is comprised of several factors that are responsible for the post-transcriptional regulation of REST expression. Two main players include beta-transducin repeat containing E3 ubiquitin protein ligase (B-TRCP) and S-phase kinase-associated protein 1 (SKP1). Previous work has demonstrated that reduction of B-TRCP increases REST stability that can contribute to oncogenic transformation of epithelial cells and tumor growth of MBs (47, 71, 72).

From these and other studies highlighting the oncogenic role of REST, therapeutic strategies against REST and its cofactors have been developed. Experiments evaluating the efficacy of pharmacological inhibitors of HDAC1/2, G9a, LSD1, and the REST complex itself have been reported in the literature (47, 73, 74). Inhibitors targeting epigenetic modifiers such as histone deacetylases (HDAC), histone methyltransferases (HMT), and histone demethylases (HDM) have proven interesting potential targets in the hunt for targeted treatments. Mutations in chromatin remodeling enzymes occur across all MB subgroups and are promising druggable targets (43, 75). Several studies have found some anti-tumorigenic activity with HDAC inhibitors in MB cells and increased HDAC activity is established to dysregulate cell cycle (76, 77). Further work in our lab has demonstrated a critical role for G9a in repressing the expression of tumor suppressor USP37 (46, 73). The activity of the coREST complex as a component of the REST
complex has been associated with poor patient prognosis and MB progression (75). Additionally, the activity of several individual coREST complex members have been implicated in MB tumorigenesis as well, including HDACs and lysine specific demethylase 1 (LSD1). While HDAC enzymes have been extensively researched and clinical trials are ongoing with HDAC inhibitors for several pediatric brain cancers, less is understood about the role of LSD1 in MB progression. LSD1 demethylates lysine residues at position 4 and 9 on histone 3 tails (H3K4/H3K9) to canonically repress transcription. Methylation at these residues has been previously reported to be altered in MB and LSD1 itself is upregulated in both neuroblastoma and MB (78, 79).

Since the discovery of LSD1 as a potential target in MB in 2013, little follow-up has been completed clarifying its role in MB development. Previous experiments demonstrated that LSD1 expression is increased in MB tumors over normal cerebellum (78). The authors found no difference in expression between subgroups. This was surprising as it is suggested by examination of patient data that LSD1 could be most relevant as a target for patients with group 3 and SHH MB. In this study, tumors from group 3 and SHH MB patients have dramatic perturbations in H3K4 methylation consistent with overactive LSD1 activity (79). Although the above study found no difference in expression levels of LSD1 between the various subgroups, enzyme activity is harder to quantify. Further, previous studies failed to examine patient outcomes including overall survival and metastasis related to LSD1 expression.
1.4 Hypothesis and specific aims

Tumor dissemination is directly related to cell migration. A major goal of my thesis is to understand how REST contributes to MB metastasis with a special focus on how REST elevation regulates normal migration of GNPs and perturbs this developmentally-regulated process. To examine these mechanisms, I utilized a transgenic mouse model expressing REST in cerebellar GNPs (the cells of origin of SHH MB) to ask how REST is affecting normal postnatal processes. In my thesis, I also sought to understand the role of REST in MB dissemination in the context of constitutive SHH signaling.

A second goal is to assess if pharmacological targeting of the REST complex will counter these migration advantages in high-REST MB cells. Strategic targeting of REST activity is enlivened by REST’s role as a chromatin remodeler and its interactions with the coREST complex. The coREST complex contains LSD1, which has been implicated in metastasis and viability of various cancer cells.

The hypotheses of this dissertation are that (a) REST elevation increases GNP and SHH MB cell migration to contribute to MB dissemination and (b) that targeting REST-associated LSD1 will counter the infiltrative behavior of REST-high MB cells.

Specific Aim 1: To determine the consequence of REST elevation on postnatal cerebellar development and GNP migration.

Specific Aim 2: To evaluate if and how REST-dependent changes in developmental signaling pathways influence MB progression.

Specific Aim 3: To assess if LSD1 can be developed as a therapeutic target for MB.
Chapter 2: Materials and Methods
Animals

Briefly, REST\textsuperscript{TG} mice were generated by using mice expressing a human REST transgene under the NeuroD2 promoter that were crossed with Math1-Cre\textsuperscript{ER} mice purchased from Jackson labs. To induce CRE-expression tamoxifen (Sigma-Aldrich) was dissolved in a foil-wrapped falcon tube with corn oil at a concentration of 20 mg/ml overnight at 37°C and then stored at 4°C for the duration of injections. Mice were injected intraperitoneally with 75 mg tamoxifen/kg body weight from postnatal day 3 to 5. The injection site was cleaned before and after injection with 70% ethanol wipes and mice were observed following days for tolerance to treatment.

Xenograft Experiments

All procedures were done under the guidelines of approved protocols through the The University of Texas M.D. Anderson Cancer Center’s (MDACC) institutional animal care and usage committee (IACUC). Mice were dually-housed, when possible, and maintained at the animal housing facility on south campus of MDACC. Immuno-compromised non-obese diabetic (NOD) severe combined immunodeficiency (scid) with a null mutation in the interleukin2 receptor gamma chain (NOD scid gamma - NSG) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Due to these three genetic features, NSG mice have reduced innate and adaptive immunity and blocked NK cell differentiation. This absence of immunity allows for the efficient grafting of foreign human cancer cells. Daoy MB tumor cells tagged with firefly-luciferase were injected intracranially following previously established protocol. Briefly, 50,000 cells were injected into groups of mice by stereotactic injection using a Harvard Apparatus infusion pump. Cells were injected through a 31-gauge burrhole (2mm caudal and 2mm lateral of the
lambdoid suture) at a rate of 0.5 ul/minute in a total volume of 3 ul. Mice were subjected to bioluminescence imaging weekly and were sacrificed at 3 months post-injection. Sacrificed animals were harvested for brain tissue.

**Bioluminescence Imaging**

In vivo imaging system (IVIS) analysis of tumor cell growth was monitored in vivo by IVIS Spectrum imaging systems. Imaging was performed in the Small Animal Imaging Facility of UTMDACC south campus. 10 minutes previous to imaging, mice were injected intraperitoneally with 150 µL of D-luciferin potassium salt diluted at 15 mg/mL in phosphate-buffered saline (PBS). Mice were anesthetized with isoflurane 5 minutes after D-luciferin injection and placed within IVIS Spectrum. Luminescent images of tumor cells were captured and analyzed using the Living Image 3.2 software.

**Patient Sample Analysis**

MB patient samples were analyzed from a publicly available NIH database (accession number: GSE85217), which is previously published. Expression values were available as Log-2 Robust Multi-array Average (RMA) from Affymetrix. These values were either normalized using z-score conversion or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression values across each patient sample. Expression values were subjected to correlation analyses and patient outcomes (where available) were matched to expression values. Heatmaps, correlation coefficients, and volcano plots were generated using GraphPad Prism.
**Cell Culture**

Human MB cell lines Daoy, UW426, D283, D341, and UW288 were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured and maintained in either Modified Eagle Medium (MEM) or Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 5% non-essential amino acids. Primary progenitor cells were harvested from wild-type and REST\(^{\text{TG}}\) mice. Progenitor cells were maintained and cultured in neurobasal media and expanded using SHH activation.

**Viral Transduction**

Short-term transductions with shLSD1 and LSD1 K661A plasmids were completed according to standard protocol. For virus generation, 293T cells were cultured in DMEM media and transfected with lipofectamine/opti-MEM and virus plasmids containing shRNA for LSD1 or LSD1 with K661A mutation. Media was changed after 4 hours and media was harvested at 24 and 48 hours and then combined. Collected media was filtered with a 70 um syringe filter and centrifuged at 1500 rpm for 5 minutes. Supernatant containing virus was stored at -80 degrees Celsius. Stable isogenic cell pairs were generated using lentiviral vectors containing human REST transgene tagged with green fluorescent protein (GFP). Cells were then sorted by fluorescence-associated cell sorting (FACS) and/or treated with puromycin (when plasmids contained puromycin N-acetyl-transferase (PAC) to confer puromycin toxicity resistance to cells). FACS sorting was completed in the Flow Cytometry and Cellular Imaging Core (MDACC) on a Becton Dickinson FACS-Aria II at an emission wavelength of 488nm in the FITC channel for GFP-positive cells.
Sorted cells were cultured and then separated and frozen into stock cultures at low passage.

**RNA-sequencing analyses**

Progenitor samples from WT and REST\textsuperscript{TG} mice were sent to Active Motif for RNA-sequencing. Functional analysis was completed using Ingenuity Pathway Analysis. Sequence alignments of RNA-sequencing data was completed using Integrative Genomic Viewer from Broadstone institute.

**Immunohistochemistry (IHC)**

Paraffin-embedded patient-derived orthotopic xenograft (PDOX) samples were provided by the lab of Xianon-Li at Texas Children’s Hospital and experiments were completed by Lin Qi. Primary mouse brain samples were harvested from mice as after proper euthanasia and sacrifice procedures. Brains were preserved in formalin and paraffin-embedded for either sagittal or horizontal sectioning. Sections were deparaffinized, dehydrated, and stained according to standard protocol. The following primary antibodies (Dilution, Catalog number Company) were used:

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<th>Dilution</th>
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<tr>
<td>GFAP (1:50 3670 CST)</td>
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<td>Vimentin (1:200, ab92547 Abcam)</td>
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<tr>
<td>GFAP (1:50 3670 CST)</td>
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<tr>
<td>Laminin (1:200, ab11575 Abcam)</td>
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<tr>
<td>CXCR4 (1:250 ab124824 Abcam)</td>
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<tr>
<td>TUBB3 (1:250 801201 Biolegend)</td>
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<tr>
<td>SDF1 (1:100 MAB350 RnD Systems)</td>
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<tr>
<td>NeuN (1:200 ab13938 Abcam)</td>
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<td>Pax-6 (1:200, PRB-278P Biolegend)</td>
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<tr>
<td>LSD1 (1:200, ab Abcam)</td>
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</table>
Secondary rabbit and mouse antibodies in glycerol were obtained from Jackson Labs and used at 1:100. Slides were counterstained with hematoxylin. All samples were completed in triplicate. Positive controls using other normal mouse organs and internal controls were used where applicable.

**Western Blot**

Primary progenitor cell and human cell samples were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors for 30 minutes with intermittent vortexing. Protein samples were subjected 4-12% sodium dodecyl sulfate-gradient gel electrophoresis and standard western blot protocol. Antibodies were diluted in TBS-T with 0.05% milk. The following primary antibodies (Dilution, Catalog number Company) were used:

<table>
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<th>Primary Antibody</th>
<th>Dilution, Catalog number Company</th>
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<tr>
<td>REST (1:1000, Millipore 07-579)</td>
<td>P-ERK (CST #9101)</td>
</tr>
<tr>
<td>Vimentin (1:200, ab92547 Abcam)</td>
<td>CXCR4 (1:250 ab124824 Abcam)</td>
</tr>
<tr>
<td>Type III b-tubulin (Abcam 1:1000),</td>
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</tr>
<tr>
<td>Actin (Abcam 1:50,000)</td>
<td>Total ERK (CST #9102)</td>
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<tr>
<td>H3K4-2me (Upstate 07-030)</td>
<td>LSD1 (1:200, ab Abcam)</td>
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<tr>
<td>H3K9-2me (Upstate 07-441)</td>
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Secondary antibodies against rabbit and mouse were purchased from Jackson labs, used at 1:100 dilution, and stored 1:1 in glycerol and stored at -20 degree Celsius. Blot development was completed using a Bio-Rad ChemiDoc imaging system. Volume densitometry was completed using the BioRad companion software.

**Quantitative Reverse Transcriptase Real-Time Polymerase Chain Reaction (qRT-PCR)**

RNA was isolated from cells using Qiagen RNA mini-prep kits. cDNA was made from isolated RNA using iSCRIPT and qRT–PCR reactions were performed following standard protocol in 96-well plates using a Roche LightCycler 96 machine. Relative mRNA expression was calculated using the comparative ΔΔ-Ct (cycle threshold) method and was normalized using 18S expression.

The following primer sequences were used (if prefaced with ‘m’, mouse; if prefaced with ‘h’, human):

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<th>Reverse Sequence</th>
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<td>GCTTTAGTCTCCGCGCCAC</td>
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<tr>
<td>mCXCR4</td>
<td>CTGTAGAGC GAGTGGTGC</td>
<td>CCAATCCATTGCCGACTA</td>
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<td>GGCCTCACTAAACCACATCAA</td>
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<td>hCXCR4</td>
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<tr>
<td>h18S</td>
<td>CGGCAGACCACCATCAGA</td>
<td>GAATCGAAACCCTGATCCCAGTC</td>
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</table>
### Scratch Test (Would closure experiment)

Human MB cells were plated in equal numbers in a 6-well plate. Cells were allowed to settle until confluent and wound was administered with a sterile tip. If applicable, drug was administered immediately following wound administration with a change of media. Images were taken of the scratch every 6 hours. Percent change of wound confluence was calculated using TimeScratch software developed by the Koumatkos group. Each well was compared to its own starting time point and measured as a percentage of confluence.

### Boyden Chamber

24-well transwell Matrigel migration assay plates and control inserts were purchased from Corning (Catalog #354480). Briefly, approximately $2.5 \times 10^4$ cells were seeded in 8 uM pore-size transwell chambers. The bottom chamber was filled with 20% FBS or SDF1 to act as a chemoattractant. Cells were set to incubate for 22 hours and cells were fixed and dyed by Hemistat triple pack and imaged under a microscope. Cell nuclei were counted from 4x images were taken of the membrane insert. CellCounter software was used to automate the counting process. Cells were plated in triplicate and averaged.
Chapter 3:

Analysis of cytoarchitectural defects in the cerebella of transgenic REST mice.
3.1 Characterization of defects

Generation of the transgenic REST mouse model

Previous studies, including our own, have implicated REST as a driver of MB progression and poor patient prognosis (47, 74). In order to investigate the specific contribution of REST to aggressive tumor behaviors, we generated a novel REST-transgenic mouse (REST\textsuperscript{TG}) (Figure 1). Briefly, REST-NeuroD2 mice were crossed with Math1-cre mice to create a tamoxifen-inducible high-REST expressing mouse with specific upregulation of REST in the cerebellar granule cell compartment, the cell of origin for SHH group MBs (8, 31, 32). The NeuroD2 specification targets cells as they are entering the inner EGL of the cerebellum to begin the differentiation process. This promoter was chosen for specificity but also provides an interesting window to see what happens when REST is elevated ectopically in a cell that is programmed to differentiate with other mechanisms. REST\textsuperscript{TG} mice are tamoxifen induced from postnatal day 3-5 to induce REST expression in the Math1\textsuperscript{+}/NeuroD2\textsuperscript{+} cellular compartment.

Examination of postnatal survival of transgenic mice revealed that a subset of mice died within a few months. The cause of death of these mice is still under investigation and tumor development did not appear to contribute to mortality. The fact that REST expression in these mice did not result in generation of tumors is consistent with previous results concluding that multiple genetic “hits” are necessary to induce tumorigenesis, including MYC amplification or overexpression.
REST elevation disrupts normal cytoarchitecture in the postnatal cerebellum

Careful analysis of the brains from REST\textsuperscript{TG} animals revealed a peculiar phenotype. Hematoxylin and eosin (H&E) staining of horizontal sections from REST\textsuperscript{TG} mice demonstrated the existence of asymmetric foliation defects in the cerebellum (Figure 12A-C).

To further investigate this, a mathematical equation was developed to quantify the symmetry between hemispheres. Normalization to hippocampal symmetry helps control for slicing error. The equation is as follows:

\[
\text{Mirror Differences} = \frac{(ACb_1 - AHP_1)}{ACb_1} - \frac{(ACb_2 - AHP_2)}{ACb_2}
\]

The conformity of the cerebella from REST\textsuperscript{TG} and wild-type (WT) animals were analyzed. Three out of the five cerebella from REST\textsuperscript{TG} mice had discernable foliation defects, and as a whole exhibited a two-fold increase in measured asymmetry. However, when two REST\textsuperscript{TG} animals with less conspicuously altered brains were excluded from the analysis, the asymmetry quotient became 3 to 4 times that of wild-type animals.

To improve the field of view for migration defects and better examine cerebellar lamination, brains were cut in the sagittal plane for staining and analysis. Wild-type mice sections stained with H&E exhibited normal lamination into the external granule cell layer (EGL), molecular layer (ML), and internal granule cell layer (IGL). H&E staining of sagittal sections of REST\textsuperscript{TG} mice brains revealed several areas of perturbed cytoarchitecture with varying degrees of severity (Figure 13).
To determine if the defects in cerebellar cytoarchitecture of \( \text{REST}^{\text{TG}} \) mice were due to changes in cell migration, IHC analyses were performed using anti-REST and anti-Vimentin antibodies. Stronger REST positivity was observed in the inner EGL cells in \( \text{REST}^{\text{TG}} \) mice compared to WT mice (Figure 13). Cells with higher REST expression in the EGL of \( \text{REST}^{\text{TG}} \) mice displayed an abnormal migration pattern and appeared to surround low-REST or REST-negative cells. Nests of cells with moderate cytoplasmic expression of REST were also seen in the cerebellar meningeal space of \( \text{REST}^{\text{TG}} \) mice, which were absent in WT mice. Vimentin immunostaining revealed disordered projections in cerebellar EGL sections where REST-high progenitors reside. Nests of cells with cytoplasmic REST expression were also highly Vimentin positive in the meninges. Together, these data capture the potential capacity of cellular REST expression to alter cell migration and agitate the postnatal cytoarchitecture of the cerebellum.

**REST elevation affects migration and organization of neuronal and glial cells**

IHC analyses were performed to determine the identity of the misplaced cells in the cerebella of \( \text{REST}^{\text{TG}} \) mice. Staining with an anti-laminin antibody was done to confirm the integrity of the basement membrane around the cerebellum and within the fissures between folia in WT mice (Figure 14). Interestingly, laminin staining of \( \text{REST}^{\text{TG}} \) mice revealed a disorganized inundation of the meninges into the cerebellar structure. The severity of basement membrane disorganization was consistent with the acuteness of the migration defect (Figure 14).

Next, utilizing the \( \text{REST}^{\text{TG}} \) mouse hemagluttinin tag (HA), we observed HA-positive cells were found in the meningeal space invaginated within and around the cortex of \( \text{REST}^{\text{TG}} \) mice (Figure 15). While not all of these cells were HA-positive, cells within the
population have clear cytoplasmic staining. This results raises an interesting possibility that REST\textsuperscript{TG} GNPs may be herding or influencing the migration of other seemingly normal cells. Further, the leptomeningeal dissemination of these cells could have significant implications in tumor progression and metastasis.

Cells of neuronal and glial origin are the most prominent players in cerebellar migration and development (10, 13, 22, 101). To ask if REST elevation disturbed migration of granule neural progenitors (GNPs) themselves, IHC analyses were performed using anti-NeuN, -beta-tubulin III (TUBB3), and -Pax-6 antibodies (for neuronal nuclei, projections, and GNPs respectively). We observed that misplaced cells were largely Pax-6 positive and negative for both NeuN and TUBB3, suggesting that these cells were indeed undifferentiated progenitors (Figure 16, Figure 17).

Interestingly, staining for glial fibrillary acidic protein (GFAP) was diminished in areas with the most pronounced migration defects (Figure 16, Figure 17). It is well established that glia are vital to scaffolding GNPs in directed migration to the IGL (51-53). Consistent with this, staining against brain lipid-binding protein (BLBP), a marker for Bergmann glia, revealed that projections in these areas were disorganized as well (Figure 16, Figure 17). There was no detectable staining for GFAP or BLBP in meningeal pockets of infiltration. These findings indicate that REST elevation in GNPs is associated with not only changes in GNP migration but also related to altered presence of glial cells and potentially their organization in the cerebellum.
Figure 11. Generation of transgenic REST mouse model. A) REST$^{TG}$ mice were created by crossing a mouse expressing a human REST transgene driven by a 1 Kb region of the NeuroD2 promoter with a tamoxifen inducible Math1-CreER mouse. B) The transgene expression is based on Cre-lox method of recombination and is tagged with 3HIS-3HA tag. PCR validated expression of the REST transgene. C) qPCR performed with primers against human REST demonstrated successful expression of the transgene in REST$^{TG}$ mice but not WT mice. D) IHC of REST$^{TG}$ animals show increased expression of REST protein in the EGL compared to WT. Data adapted from work done for another unpublished manuscript in the lab. Work and figure produced by Tara Dobson.
Figure 12. Horizontal brain slices taken from Wild-type and REST$^{TG}$ mice. Horizontal sections were taken from postnatal 14 day old mice and analyzed for cerebellar symmetry. Brains from REST$^{TG}$ mice appeared to display a midline shift. A) Panel of horizontal sections from WT and REST$^{TG}$ mice. B) Example of increased magnification of asymmetry. C) Symmetry equation $A=\text{Area of, Hp=Hippocampus, Cb=Cerebellum (n=3, n=5; p<0.05).}$
Figure 13. REST elevation disrupts normal cytoarchitecture in the postnatal cerebellum. (A) Sagittal sections stained with H&E from p8 WT and REST$^{TG}$ mice. (B) Vimentin and REST immunostaining of WT and REST$^{TG}$ mice. (C) Lower magnification images of REST and Vimentin staining.
Figure 14. Laminin staining of basement membrane. A) Staining comparison of anti-laminin showing compromised meningeal structure and inundation of basement membrane. B) Quantification of parenchymal laminin staining (n=3; *p<0.05; **p<0.01).
Figure 15. Pockets of expanded, ectopically placed cells contain HA-positive GNPs.

Analysis of sagittal sections of WT and REST$^{TG}$ mice revealed the existence of pockets of misplaced cells that contain hemaglutinin-positive cells in REST$^{TG}$ mice.
Figure 16. REST elevation affects migration and organization of neuronal and glial cells. A) Representative images at 40x magnification of NeuN staining, TUBB3 staining and Pax-6 staining (n=3, top panel). B) Representative images at 40x magnification of BLBP staining and GFAP staining of p8 WT EGL areas and REST$^{TG}$ mice abnormal EGL areas (n=3, bottom panel). Black arrows point to areas of interest, misplaced cells in the parenchyma or meningeal space.
Figure 17. Alternative objectives: REST elevation affects migration and organization of neuronal and glial cells. Representative images at A) 10x magnification and B) 100x magnification of NeuN staining, TUBB3 staining, Pax-6 staining, BLBP staining, and GFAP staining of p8 WT EGL areas and REST\textsuperscript{TG} mice abnormal EGL areas (n=3). Black arrows point to areas of interest, misplaced cells in the parenchyma or meningeal space.
3.2 Identification and validation of crucial gene expression changes

REST elevation increases CXCR4 activation and chemokinesis of granule cells to SDF1

We next examined the mechanism by which REST influences GNP function and lineage specification. In order to search for candidates, RNA-sequencing of GNPs from WT and REST\textsuperscript{TG} mice was performed. Analysis of RNA-sequencing data using ingenuity pathway analysis revealed 572 significant differentially expressed genes (Figure 16). Pathway analysis of the RNA-sequencing data identified significant alteration of genes involved in cell. Surprisingly, a number migration related genes were downregulated in REST\textsuperscript{TG} mice compared to WT mice. (Figure 18).

More careful analysis of the data illuminated a few crucial expression changes in molecular players of proper cerebellar lamination BDNF, CXCR4, and SDF1 (Figure 19). CXCR4 signaling is of critical importance to proper development of the cerebellar EGL and depletion of CXCR4 can lead to foliation defects associated with early radial migration (18, 39).

Transcriptome comparisons between WT and REST\textsuperscript{TG} mice revealed a variable 5 to 10 fold increase in REST gene expression. This elevation was associated with increased CXCR4 and SDF1 transcript expression, with the largest and most significant effect occurring in the animal with the highest REST (Figure 20). GNPs from these mice had reduced differentiation as shown by reduced beta-tubulin class III (TUBB3) expression and increased GFAP expression suggestive of retrograding lineage specification. REST repression of BDNF was confirmed in these GNPs as well.
When compared with WT GNPs at the protein level, GNPs from REST\textsuperscript{TG} mice had a 3-fold increase in CXCR4 protein and a 2-fold increase in SDF1 protein (Figure 21). Interestingly, while activation of downstream effector of CXCR4, phosphorylated ERK1/2, (p-ERK – normalized to total ERK) was increased 3-fold, total ERK 1/2 was also elevated 2-fold (Figure 21). These changes appear to be post-transcriptionally as ERK 1 and ERK 2 levels are not similarly increased at the transcript level. A two-fold increase in expression of vimentin is seen in GNPs from REST\textsuperscript{TG} mice, indicating changes in GNP motility in response to REST protein upregulation.

In line with increased expression of CXCR4 protein, GNPs from REST\textsuperscript{TG} mice exhibited a 3-fold increase in migration to SDF1 as compared to GNPs from WT mice (Figure 21).

IHC of cerebella from REST\textsuperscript{TG} mice revealed moderate staining of CXCR4 with less organized patterning in the parenchyma compared to sections from WT mice (Figure 22, 23). Indeed, CXCR4 staining was very dense in REST\textsuperscript{TG} mice in areas of GNP expansion near the meningeal barrier. Compared to WT samples, SDF1 immunostaining was also increased in REST\textsuperscript{TG} mice, especially in cell pockets of expanded meningeal space (Figure 22).
Figure 18. RNA sequencing of GNPs from WT and REST$^{TG}$ mice. RNA sequencing of GNPs from WT and REST$^{TG}$ mice was completed by Active Motif (Carlsbad, CA). Functional analysis revealed several significantly changed functions including cancer, cellular movement, and development. A) Volcano plot of significantly altered gene expression (green dots = p<0.05; red dots = q<0.05). B) Ingenuity Pathway Analysis functional analysis.
Figure 19. RNA-sequencing alignments with CXCR4, SDF1, and BDNF genes. Integrative genome viewer (IGV) alignment of RNA-sequencing data with CXCR4, SDF1, and BDNF genes revealed that GNPs from REST\textsuperscript{TG} mice altered the expression of these lamination-important genes.
Figure 20. Differential transcript expression of GNPs from REST\textsuperscript{TG} mice. GNPs from WT and REST\textsuperscript{TG} cells were analyzed for transcript levels of REST, CXCR4, ERK1, ERK2, SDF1, BDNF, TUBB3, and GFAP. All transcripts were normalized to 18S and then WT gene expression. (n=3; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).
Figure 21. Differential protein expression and chemokinetic activity of GNPs from REST\textsuperscript{TG} mice. A) GNPs from WT and REST\textsuperscript{TG} cells were subjected to western blot to assess protein levels of REST (band at ~120 kDa), CXCR4 (band at ~40 kDa), phosphorylated ERK (p-ERK, band at ~42 kDa), total ERK (band at ~42 kDa), SDF1 (band at ~30 kDa), and vimentin (band at ~54 kDa). Total H3 served as a loading control (band at ~20 kDa). B) Quantification of blot (n=3; *p<0.05, **p<0.01). C) Migration of GNPs from REST\textsuperscript{TG} mice was significantly 3-fold higher than progenitors from WT mice (n=3; p<0.05). D) Transcript of ERK1/2 was minorly but significantly altered by REST elevation in GNPs.
Figure 22. Immunohistochemistry of CXCR4 and SDF1 in sagittal brain sections from WT and REST\textsuperscript{TG} mice. Representative images at 40x magnification of immunostaining of CXCR4 and SDF1 which is increased in areas of meningeal invagination and ectopically placed cells.
Figure 23. Alternative objectives: Immunohistochemistry of CXCR4 and SDF1 in sagittal brain sections from WT and REST<sup>TG</sup> mice. A) Representative images at 10x and 100x magnification of immunostaining of CXCR4 and B) SDF1.
In summary, this chapter has focused on describing how postnatal induction of REST expression perturbs postnatal cerebellar development. We observed that REST expression alone did not induce tumor formation. However, REST expression in the GNP compartment did alter the normal cerebellar cytoarchitecture. These abnormalities were observed in the GNP compartment and the glial compartment and were accompanied by an altered laminin/basement membrane structure. Molecular analysis of GNPs with transgenic REST expression revealed a number of differentially expressed genes. Among them were genes of critical importance to proper cerebellar lamination, including BDNF, SDF1 and CXCR4. As BDNF is a known REST target, we focused on CXCR4 – a novel interaction for REST. REST increased CXCR4 expression at the protein and transcript level, however changes at the protein level were more significant and consistent. Furthermore, GNPs from REST\textsuperscript{TG} mice exhibited a significantly stronger response to the CXCR4 ligand, SDF1, as measured by elevated downstream ERK signaling. Taken all together, the results from chapter 1 suggest that REST is capable of perturbing the CXCR4 signaling axis to regulate mobility of GNPs during neuronal maturation. A graphical summary of the results and their interpretation is shown in figure 24.
Differentiation Pathways

Radial Migration
Meningeal Integrity

REST

Proliferation
Tangential Migration
Overexpression of REST in cerebellar GNP results in abnormal cytoarchitecture of the cerebellum. Normal cerebellar lamination is ensured by temporal expression of CXCR4 in the GNP of the outer EGL. These cells migrate tangentially to SDF1 secreted by meningeal cells. As cell begin the differentiation process, cell responsiveness to CXCR4 stimulation is blocked (EphrinB2 expression is postulated to play a role in this) and cells begin responding to glia-associated radial migration cues. GNP finish their migration by performing glia-independent migration past the PL, to their final destination in the IGL where they become mature granule neurons with projections spanning from the EGL to the internal white matter tract. In our REST\textsuperscript{TG} model of abnormal cerebellar development, elevated CXCR4 expression in GNP cells may be responding to disorganized SDF1 secretion in the EGL resulting in an abnormal retention of GNP in the EGL. This retention would be strengthened by blocked radial migration mechanisms (BDNF, EphB2, and glial organization) leading to increased proliferation and tangential migration in the outer EGL.

EGL – external granule cell layer; ML – molecular layer; PL – Purkinje layer; IGL – internal granule layer; SDF1 (green dots) – stromal derived factor 1; BDNF (pink dots) - brain derived neurotrophic factor 1;
Chapter 4:

REST and SHH signaling cooperatively upregulate CXCR4 in disseminated MB
4.1 Sonic hedgehog medulloblastoma mouse models \textit{in vivo}

It is established that REST expression can promote MB growth, progression, and aggressive disease course. It is also established that CXCR4 expression is related to metastasis and worsened patient prognosis. This effect is especially prominent in patients with SHH MB. However, the cooperation of REST and CXCR4 in the context of cancer has not been reported previously. The results of the experiments in REST$^{TG}$ mice described in the previous chapter suggest that REST increases CXCR4 expression and chemotactic responsiveness to SDF1. However, whether or not these mechanisms can operate in the context of MB are unknown.

The identification of GNPs as the cell of origin of SHH MB and the availability of transgenic mouse models has advanced our understanding of molecular mechanisms driving tumor behaviors in the context of SHH hyperactivity. Further, REST is a demonstrated driver of aggressive tumor behaviors in SHH MB but the mechanisms of how REST promotes tumor dissemination are still being investigated. We propose that REST and the SHH pathway cooperatively upregulate CXCR4 signaling, which is known to drive MB dissemination and metastasis. To investigate this, we utilized our transgenic REST mouse model along with a popular mouse model of SHH MB (Ptch$^{+/}$-). This mouse lack an allele for the PTCH1 receptor. This deregulates the SMO receptor and allows for increased SHH signaling through Gli activator transcription factors. This Ptch$^{+/-}$ mouse was crossed with our REST$^{TG}$ mouse to create a mouse with high levels of REST in the context of constitutive SHH activation (REST$^{TG}$/Ptch$^{+/-}$). Tumors from these mice were immunohistochemically examined for CXCR4 expression and downstream activity.
REST elevation in the context of constitutive SHH signaling enhances CXCR4 signaling

Work from other groups has previously established that mice with tumors stemming from constitutive SHH activity in GNPs had elevated CXCR4 expression (39, 42, 80-82). Mechanisms for upregulation of CXCR4 have been demonstrated to be due to loss-of-function mutations in negative regulators of CXCR4 like G protein-coupled receptor kinase 5 and 6 (GRK5/6) (83). GRK5/6 is a kinase with downstream activity that helps internalize CXCR4. It is already established that GRK5/6 is inhibited by an oncogene named WIP1 (84). However, analysis of our RNA-sequencing data did not reveal any significant differences in GRK5/6 or WIP1 transcript expression between WT and REST\textsuperscript{TG} mice. However, we cannot exclude changes in protein levels.

Having found that REST elevation increased CXCR4 expression and signaling, we aimed to determine if this REST-dependent amplification would increase tumor dissemination. As described in the previous chapter, REST\textsuperscript{TG} mice do not form tumors, but exhibit defects in GNP migration and cerebellar architecture. Ptch+/- mice do develop tumors by 6-9 months and at a frequency of 15-20%. Interestingly, REST elevation in the context of Ptch haploinsufficiency (REST\textsuperscript{TG}/Ptch+/-) caused tumor formation in 100% of the mice between 10-90 days. Survival of Ptch+/- and REST\textsuperscript{TG}/Ptch+/- mice revealed that REST\textsuperscript{TG}/Ptch+/- mice had drastically reduced survival compared to Ptch +/- mice (Figure 25). Importantly, while there was no overt evidence of spinal metastasis, tumors from these mice exhibited highly infiltrative disease compared to the localized tumors in Ptch+/- mice.
To examine the activity of the CXCR4 signaling cascade in this SHH tumor environment, Ptch+/- and REST\textsuperscript{TG}/Ptch+/- mice were sacrificed after significant symptoms of tumor burden were exhibited (representative images were taken from mice sacrificed at ages day 45 and day 239, respectively). IHC analyses of REST\textsuperscript{TG}/Ptch+/- tumor samples revealed increased REST and Vimentin staining density compared to Ptch+/- tumors (Figure 26, Figure 28). CXCR4 and SDF1 staining was also elevated in REST\textsuperscript{TG}/Ptch+/- tumors (Figure 27, Figure 28). While both tumors were CXCR4 positive, REST\textsuperscript{TG}/Ptch+/- tumors exhibited substantially increased CXCR4 positivity, especially around areas of meningeal invagination (Figure 27, Figure 28). Immunostaining of downstream effector of CXCR4 signaling, p-ERK, was more pronounced in the REST\textsuperscript{TG}/Ptch+/- tumor samples (Figure 27, Figure 28). BDNF staining revealed heterogenous expression in Ptch+/- tumors but was largely undetectable in REST\textsuperscript{TG}/Ptch+/- tumors (Figure 26, Figure 28).

Overall, these histological analyses show that REST and SHH signaling cooperate in decreased BDNF and increased CXCR4/SDF1 levels and enhanced signaling through the CXCR4 receptor. These immunostaining patterns are correlated with dramatically reduced survival and leptomeningeal dissemination.
Figure 25. Survival curve analysis of Ptch+/− and REST<sup>TG</sup>/Ptch+/− mice. A) REST<sup>TG</sup>/Ptch+/− mice were generated by crossing REST<sup>TG</sup> with Ptch+/− mice. B) Survival of REST<sup>TG</sup>/Ptch+/− mice was drastically reduced compared to WT and Ptch+/− mice and C) tumor burden was larger. D) H&E staining of tumors from REST<sup>TG</sup>/Ptch+/− mice showed that these tumors had rougher tumor edges indicative of more infiltrative tumors.

Figure adapted from manuscript currently under review describing REST deregulation of SHH signaling. Work and figure produced by Tara Dobson and Rong-Hua Tao.
**A**

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**B**

- **REST**
  - Ptch<sup>+/−</sup> vs. REST<sup>TG</sup>/Ptch<sup>+/−</sup>: p = 0.02711
- **Vimentin**
  - Ptch<sup>+/−</sup> vs. REST<sup>TG</sup>/Ptch<sup>+/−</sup>: p = 0.08162
- **BDNF**
  - Ptch<sup>+/−</sup> vs. REST<sup>TG</sup>/Ptch<sup>+/−</sup>: p = 0.01139
Figure 26. 40x images of immunohistochemistry of REST, Vimentin, and BDNF in MB tumors from Ptch+/- and REST^{TG} mice. A) Representative images at 40x magnification of REST, Vimentin, and BDNF immunostaining in Ptch +/- (n=4) and REST^{TG}/Ptch+/- (n=3) tumors. B) Quantification of staining density was completed under blinded conditions. Five fields of 40x images per tumor were taken for analysis.
A. CXCR4, SDF1, p-ERK

B. Graphs showing evaluated staining density for CXCR4, SDF1, and p-ERK.
Figure 27. 40x images of immunohistochemistry of CXCR4, SDF1, and p-ERK in MB tumors from Ptch+/- and REST<sup>TG</sup> mice. A) Representative images at 40x magnification of CXCR4, SDF1, and phosphorylated-ERK1/2 (p42/44) immunostaining in Ptch +/- (n=4) and REST<sup>TG</sup>/Ptch+/- (n=3) tumors. B) Quantification of staining density was completed under blinded conditions. Five fields of 40x images per tumor were taken for analysis.
Figure 28. 4x and 10x images of immunohistochemistry of MB tumors from Ptch+/- and REST\textsuperscript{TG} mice. REST elevation in the context of constitutive SHH signaling enhances CXCR4 signaling. A) Representative images at 4x and 10x magnification of REST, Vimentin, and BDNF immunostaining of Ptch+/- and REST\textsuperscript{TG}/Ptch+/- tumors. B) Representative images at 4x and 10x magnification of CXCR4, SDF1, C) phosphorylated-ERK1/2 (p42/44) and BDNF immunostaining of tumors.
4.2 Human medulloblastoma patient data

Validation of REST-CXCR4 connection in SHH MB patient samples

In order to validate the clinical relevance of the REST-dependent elevation on CXCR4, we examined publicly available MB patient data sets provided by the St. Judes Legacy Genomes Project (NIH GSE34217 and the National Institute of Health (NIH GSE85217) (7).

Interestingly, primary analysis of patient samples from the St. Judes dataset found that expression of REST expression did not significantly differ between groups (Figure 29). However, a negative post-transcriptional regulator of REST, beta-TRCP, was lowest in SHH MB. Further, expression of beta-TRCP’s complex member, S-phase kinase-associated protein 1 (SKP1), was also very low in SHH. The downregulation of these essential components of the SCP (SKP1-CUL1-F-box protein) ubiquitin ligase complex members suggest that stabilization of REST protein is potentially increased in these patients. This was associated with an increase in CXCR4 expression in SHH MB (Figure 30).

These were some preliminary results suggesting that REST and CXCR4 may have a cooperative relationship in SHH MBs, especially.

In the Cavalli et al datasets, we found that REST and CXCR4 expression was significantly correlated across all 763 MB patient samples (Figure 31). Examination of correlations between REST and CXCR4 expression across MB molecular subgroups uncovered a subgroup-dependent effect of correlation (Figure 32). The WNT subgroup of MB had a negative but insignificant correlation between REST and CXCR4 transcript
expression. Groups 3 and 4 had significant positive correlations between REST and CXCR4 transcript expression with correlation coefficients of $r = .25$ and $r = .36$ respectively.

Interestingly, despite the description of CXCR4 upregulation in SHH MB mouse models, we failed to observe a significant correlation between REST and CXCR4 across SHH MB subgroups. However, closer analysis based on the newly reported subclassification of SHH MBs into $\alpha$, $\beta$, $\Gamma$, and $\delta$ identified strong and significant correlation coefficients of $r = .42$ in SHH $\beta$ and $r = .46$ in SHH $\delta$ subtypes. Out of these two groups, SHH $\beta$ patients had the highest levels of REST and CXCR4 and also had a significant correlation with CXCR4 ligand, SDF1 (data not shown).

**Division of SHH MB patient samples based on REST and CXCR4 expression**

Focusing on the SHH subgroup, we put the patient samples into four conditions, based on REST and CXCR4 expression (Figure 33). The conditions were determined as follows: REST and CXCR4 transcript expression both being higher than average (both high), REST transcript expression being lower than average and CXCR4 transcript expression being higher than average (REST low, CXCR4 high), REST transcript expression being higher than average and CXCR4 transcript expression being lower than average (REST high, CXCR4 low), and REST and CXCR4 transcript expression both being lower than average (both low).

When we examined patient outcomes based on these conditions, we observed the majority of patients with metastasis within the SHH- $\beta$ and $\delta$ groups had high transcript expression of both REST and CXCR4 (Figure 33). 100% of patients with metastasis in the SHH $\beta$ subtype had high REST levels, and 90% of those patients expressed high
CXCR4 concurrently. SHH β patients are characterized by a predominantly infantile age, metastasis, and poor prognosis (Figure 3).

Examination of overall survival across the whole SHH cohort did not reveal any differences between both high and both low groups (Figure 34). However within the SHH-beta group, there was a large but insignificant difference in the survival curve likely due to insufficient patient data in these groups.

These results are the first to implicate the importance of REST and CXCR4 cooperativity in poor patient outcomes. These results seem to be specifically relevant in patients in the SHH β subgroup, mostly infantile patients with metastatic disease.

**Examination of patient derived orthotopic xenografts (PDOX) for REST-associated CXCR4 expression and signaling**

To further examine the effect of REST elevation on CXCR4 expression and signaling, we obtained samples from Patient-Derived Orthotopic Xenograft (PDOX) models of SHH MB (Figure 35). PDOX samples were immunohistochemically analyzed for REST and CXCR4 expression and activation. PDOXs were labelled either low-REST or high-REST based on levels of REST expression (Figure 36, Figure 37). BDNF was not detectable in Low- or High-REST tumors. Vimentin positivity was observed in low-REST tumors to be along the tumor boundary, whereas that in high-REST tumors included the parenchyma as well. CXCR4 and phosphor-ERK1/2 staining was directly correlated with REST levels in these tumors and SDF1 expression was present in cells infiltrating the surrounding parenchyma.
These results further support that REST elevation contributes to an increase of CXCR4 levels and enhanced ERK1/2 phosphorylation in SHH sub group murine and human MBs.
St. Judes Legacy Genome Project expression of REST, B-TRCP, and SKP1.

Transcript expression of A) REST B) B-TRCP and C) SKP1 across MB subgroups from the St. Judes Genome Project 2016. The x-axis of these graphs contains z-scores for REST, BTRC, and SKP1 transcript probes.
Figure 30. St. Judes Legacy Genome Project expression of REST, CXCR4, and SDF1. Transcript expression of A) REST B) CXCR4 and C) SDF1 across MB subgroups from the St. Judes Genome Project 2016. The x-axis of these graphs contains z-scores for REST, CXCR4, and SDF1 transcript probes.
Figure 31. REST and CXCR4 transcript correlation across all MB. A) Expression of REST and CXCR4 are positively correlated across 763 MB patient samples. C) This Cavalli et al, 2017 dataset was the first to subtype WNT, SHH, Group 3, and Group 4 MB into α, β, γ, and δ.
A

REST and CXCR4 - WNT

r = -0.1143
p = 0.3462

REST and CXCR4 - SHH

r = 0.1102
p = 0.3822

REST and CXCR4 - Group 3

r = 0.2496
p = 0.002558

REST and CXCR4 - Group 4

r = 0.3576
p < 0.0001

B

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Figure 32. Subgroup specific correlation of REST and CXCR4 transcript expression. A) Correlations of REST and CXCR4 transcript expression in the MB subgroups. Significant correlations were observed in Group 3 and Group 4 MB. B) Further analysis of SHH subgroups revealed that REST and CXCR4 are strongly correlated in SHH β and δ groups.
Figure 33. Classification of REST and CXCR4 expression and SHH MB patient metastasis. A) SHH MB samples were stratified into groups based on CXCR4 and REST expression patterns. B) Analysis of patients with metastasis in SHH subtypes revealed that 100% of patients with metastasis in the SHH β subtype had high REST and ~90% of the same patients had CXCR4 co-elevated. The majority of patients in both α and δ also had high REST and high CXCR4 expression.
Figure 34. Survival of SHH patients with high or low REST/CXCR4 expression. A) Kaplan-Meier survival curves of patients from high and low REST and CXCR4 tumor transcript expression across all SHH patients and B) SHH-beta patients.
Figure 35. Schematic of patient-derived orthotopic xenografts procedure. These experiments were done by collaborator Xiao-nan Li at Texas Children’s Hospital. Tumor cells from patient tumors were cultured and injected orthotopically in mice. Cells that established tumors were passaged through inoculation of mice.
Figure 36. 40x objectives of low-REST and high-REST patient-derived orthotopic xenografts (PDOX). Respective images at 40x magnification of IHC of low-REST and high-REST PDOX samples. Immunostaining of A) REST, Vimentin, BDNF, B) CXCR4, SDF1, and p-ERK was completed.
Figure 37. Alternative objectives: low-REST and high-REST patient-derived orthotopic xenografts (PDOX). Respective images at 10x magnification of IHC of low-REST and high-REST PDOX samples. Immunostaining of REST, Vimentin, BDNF, CXCR4, SDF1, and p-ERK was completed.
4.3 Human medulloblastoma cell lines *in vitro*

**Examination of orthotopic xenografts for REST-associated CXCR4 expression and signaling using Daoy cells engineered with REST transgene**

In similar xenograft experiment, Daoy MB cells were transduced with a REST transgene (Daoy-REST) tagged with green fluorescent protein (GFP) and firefly luciferase (FFLUC) or a control vector with GFP and FFLUC. Daoy-REST and control Daoy cells were injected orthotopically into the cerebella of immunodeficient mice and tumor establishment was verified using bioluminescence imaging (BLI) (Figure 38). Mice were sacrificed at day 60 and tumor differences were examined.

Consistent with previous experiments, we found increased tumor burden in mice injected with Daoy-REST cells (data not shown). Compared to Daoy tumors, tumors established with Daoy-REST cells were highly infiltrative and had higher number of invasive cell nodes in the surrounding parenchyma (Figure 39).

Daoy-REST xenografts also promoted the presence of extracranial tumors that had spread into and outside of the skull (Figure 39). Both Daoy and Daoy-REST tumors exhibited similar staining density of Vimentin (Figure 40). However, samples from Daoy-REST xenografts displayed areas of vimentin positive projections that extended through the normal parenchyma and connected the primary tumor with disseminated nodes of Daoy-REST cells. As expected, Daoy-REST tumors had higher REST expression than Daoy tumors. Interestingly, extracranial tumors arising from Daoy-REST cells had elevated levels of REST compared to the intracranial tumor, suggesting that the more motile cells had increased REST.
Further, Daoy-REST tumors had higher amounts of CXCR4 expression than Daoy tumors, the highest levels being in the extracranial tumors of the Daoy-REST injected mice (Figure 41). Examination of downstream marker phosphorylated ERK1/2 revealed increased levels of signaling in both Daoy-REST tumor populations. Finally, BDNF was not detectable in Daoy or Daoy-REST tumors (Figure 40).

REST elevation and increased migration is recapitulated in MB cells in vitro and blocked by CXCR4 antagonism

To more directly assess changes in migratory capacity in response to REST elevation, in vitro scratch tests and transwell migration assays were performed using an isogenic cell pairs of human SHH MB, UW228/UW228-REST and Daoy/Daoy-REST. Daoy-REST and UW228-REST cells exhibited about a two-fold increase in migration as measured by scratch test compared to their isogenic counterpart (Figure 42). This was recapitulated in transwell migration assay, where UW228-REST and Daoy-REST cells migrated to 20% FBS twice as much as UW228 and Daoy cells respectively (Figure 43).

Functional and pathway analysis of RNA-sequencing data from isogenic cell pair Daoy and Daoy-REST revealed similar changes in gene expression compared to RNA-sequencing of GNPs from WT and REST\textsuperscript{TG} mice (Figure 44). Top functions related to increased REST expression included cancer, cell movement and adhesion, and development. Pathway analysis included a significant positive predicted z-score relating to increased CXCR4 signaling (Figure 45).

Western blot analyses confirmed a 2.10 fold increase in CXCR4 protein in UW228-REST UW228 (Figure 46). However, SDF1 levels were decreased in UW228-REST cells.
Phosphorylation of ERK1/2 was slightly increased by 1.23 fold in UW228-REST cells and a similarly small REST-dependent 1.20 fold change in total ERK levels was also observed. BDNF was undetectable in our hands (data not shown). These experiments were also carried out in Daoy and Daoy-REST cells, which already had elevated levels of CXCR4 and REST.

To examine if CXCR4 hyperactivity contributed to REST-dependent increases in migration, we treated UW228 and UW228-REST cells with the CXCR4 antagonist AMD3100 before subjecting them to a transwell migration assay with SDF1 as the chemoattractant. Untreated UW228-REST cells migrated almost two-fold more than UW228 cells on average, but did not reach significance. AMD3100 treatment caused a significant decrease in migration of UW228-REST cells and, although not significant, also reduced the migration of UW228 cells (Figure 47). AMD3100 also blocked the REST-dependent advantage in migration seen by Daoy-REST cells in a standard transwell migration assay, while Daoy cells remained largely unaffected (Figure 48). These results suggest that AMD3100 is blocking SDF1-mediated chemokinesis through CXCR4.
Figure 38. Schematic of Daoy/Daoy-REST orthotopic xenograft procedure. The in vivo work for these experiments were completed under the guidance and with the help of lab members, Bridget Kennis and Shavali Shaik. Daoy and Daoy-REST MB cell lines were cultured and injected orthotopically in mice. Tumor establishment and growth was monitored using bioluminescence imaging.
Figure 39. Hematoxylin and Eosin staining of Daoy and Daoy-REST xenografts.

Mice injected with Daoy-REST cells developed large tumor burden and extracranial tumors. H&E staining of sections of tumors from xenograft experiments allow for visualization of the tumor. Tumors established with Daoy-REST cells have rough tumor edges and a higher number of invasive nodes (black arrows).
Figure 40. Immunohistochemistry of REST, Vimentin, and BDNF in Daoy and Daoy-REST xenografts. Representative images of 10x and 40x magnification of immunostaining of REST, Vimentin, BDNF in Daoy and Daoy-REST xenografts.
Figure 41. Immunohistochemistry of CXCR4, SDF1, and p-ERK in Daoy and Daoy-REST xenografts. Representative images of 10x and 40x magnification of immunostaining of CXCR4, SDF1, AND p-ERK in Daoy and Daoy-REST xenografts.
Figure 42. REST transgene increases wound healing migration of SHH MB cell lines, UW228 and Daoy. UW228 and Daoy cells with and without REST transgene expression were subjected to scratch wound migration assay. Cells expressing REST transgene demonstrated 2-fold increase in migration (n=3; *p<0.05).
Figure 43. REST transgene increases transwell migration of SHH MB cell lines, UW228 and Daoy. A) Isogenic cell pairs of UW228 and Daoy cells were subjected to transwell migration assay. Cells expressing REST transgene demonstrated a near 2-fold increase in migration (n=3; *p<0.05). B) Invasion index normalizing cells to proliferating cells in the top chamber reveal a near two-fold increase in invasive capacity. C) Daoy-REST cells migrated significantly more than Daoy cells over time.
Figure 44. Functional analysis of RNA-sequencing data from Daoy and Daoy-REST.

Ingenuity pathway analysis of RNA-sequencing data revealed several significantly altered functions between Daoy and Daoy-REST. The top 26 significant functions are represented here.
Figure 45. Pathway analysis of RNA-sequencing data from Daoy and Daoy-REST cells. Ingenuity pathway analysis of RNA-sequencing data revealed several significantly altered signaling pathways between Daoy and Daoy-REST. Six of the top pathways of interest are represented here. CXCR4 signaling was predicted to be significantly increased in response to REST expression.
Figure 46. Isogenic cell pairs western blots. Cell lysates from isogenic cell pairs demonstrate a slight increase in CXCR4, SDF1, and p-ERK. Calcium-sensing protein Calmodulin (Calm) was also increased in UW228-REST but not Daoy-REST lysates.
Figure 47. REST-induced increase in chemokinesis to SDF1 is reduced by CXCR4 receptor antagonism in UW228 cells. Chemokinesis to 100 ng/mL of SDF1 was increased in UW228-REST cells compared to UW228, with a trend reaching significance (n=3). Migration of cells to SDF1 was blocked by administration of 2.76 uM of CXCR4 antagonist, AMD3100.
Figure 48. REST-induced increase in chemokinesis to SDF1 is reduced by CXCR4 receptor antagonism in Daoy cells. Chemokinesis to 100 ng/mL of SDF1 was significantly increased in Daoy-REST cells compared to Daoy (n=3; *p<0.05). The advantage of REST given to the migration of cells to SDF1 was blocked by administration of 2.76 uM of CXCR4 antagonist, AMD3100. However, this reduction did not completely abolish the cell migratory response to SDF1, suggesting that the cell could still respond without CXCR4. Scratch wound migration revealed no significant differences in migration between treated and untreated cells at 24 hours post-wound.
In summary, these results highlight the ability of REST to upregulate CXCR4 and promote signaling through the CXCR4 and SDF1 axis in the context of SHH MB. This is associated with leptomeningeal dissemination and reduced survival and larger tumor burden in our mouse models.

In patient samples, CXCR4 and REST are correlated in patients with metastasis and that patient-derived tumor samples with high-REST also display high levels of CXCR4 immunostaining compared to low-REST tumors. In xenograft mouse models with MB cell line Daoy, it is demonstrated that REST-transgene expression increases tumor dissemination and marker of invasive cells, vimentin. We also examine these cells in vitro and verify that REST transgene expression results in increased CXCR4 expression and downstream signaling. Further, the migration advantage to SDF1 given by the REST-transgene can be blocked by administration of CXCR4 antagonist, AMD3100.

These model of our hypothesis based on chapters 1 and 2 is referenced in figure 49.
Figure 49. Summary of hypothesis from chapters 1 and 2. REST increases the protein expression of CXCR4 to influence perturbation of GNP migration and cerebellar lamination and also cooperates with CXCR4 to increase tumor cell migration. REST also represses BDNF which has a demonstrated capability to negatively influence CXCR4.
Chapter 5:
Targeting REST-LSD1 activity in medulloblastoma
5.1 Analysis of patient data

Elevated levels of LSD1 are related to reduced survival of medulloblastoma patients.

Since the discovery of LSD1 as a potential target in MB in 2013, little follow-up has been completed clarifying the role that LSD1 plays in MB development. Previous experiments demonstrated that LSD1 expression is increased in MB tumors over normal cerebellum. The authors found no difference in expression between subgroups. This was surprising as it is suggested by examination of patient data that the relevance of LSD1 could be most important in patients with SHH and group 3 MBs. Tumors from these patients have drastic perturbations in H3K4 methylation consistent with overactive LSD1 activity (79). While previous experiments have found no difference in expression levels of LSD1 in SHH or Group 3 tumors over other subgroups, enzyme activity is harder to quantify. Further, previous studies failed to examine patient outcomes including overall survival and metastasis related to LSD1 expression.

In this chapter, we analyzed a large, publicly available patient dataset and examined patient outcomes related to LSD1 transcript expression. We also cluster SHH MB subgroups based on published LSD1 targets to examine predicted LSD1 activity. In subsets of SHH patients, mostly in the alpha subgroup, we find that patients have high LSD1 levels in conjunction with high cofactor REST expression and protein deubiquitylase USP7. Contrasting this cluster with an opposite aligning cluster revealed significant differences in patient survival. Further, we found that high REST increases the viability and migration of cells, consistent with previous reports. Genetic knockdown of
LSD1 was sufficient to block SHH MB cell growth, even in the presence of elevated REST. However, pharmacological inhibition of LSD1 had no effect on cell growth in these cell lines but did have REST-dependent effect on cell migration. In line with this, we find that irreversible LSD1 inhibition did not restore BMP2 gene expression in the way that genetic knockdown did. However, REST target HIF1A upregulation was reduced by irreversible LSD1 inhibition. These findings provide novel evidence for the differential role of LSD1 in the context of REST in invasive MB and provide a new molecular target for the REST-LSD1 cofactor complex.

Division of patient samples into high, moderate, and low expression of LSD1 revealed an effect of LSD1 expression overall survival in MB patients. Divisions were made as follows: low (Z-score < -0.40), moderate (0.40 > Z-score > -0.40), and high (Z-score > 0.40). These groups are arbitrary however, as the dataset from GSE85217 has no normal tissue control and it is already established that LSD1 expression is elevated in comparison to normal tissue.

**Patient outcomes across all MB related to LSD1 transcript expression.**

It has been previously established that LSD1 transcript and protein expression is elevated in MB tumor tissue compared to normal tissue (78). This study also found no differences in LSD1 transcript expression between subgroups. However, analysis of a publicly available dataset (GSE85217) of 763 MB tumor samples revealed a significant analysis of variance in LSD1 transcript expression (p<0.0001; Figure 50). Within this dataset, Group 4 MB samples were significantly lower than the other 3 subgroups. There were no significant differences between WNT, SHH, or Group 3.
Further, previous study did not look at patient outcomes associated with LSD1 expression. Analysis of Kaplan-Meier curves revealed early survival differences in patients with low and high LSD1 levels (Figure 51). Interestingly, Mantel-Cox analysis of survival revealed a significant difference between low and moderate LSD1 across all of the MB patients (Figure 51). Subgroup specific analysis of overall survival related to LSD1 expression found that Group 3 MBs had the most significant survival curve differences related to LSD1 expression (Figure 52). Surprisingly, the SHH subgroup did not have a significant curve difference despite previous studies noting that both SHH and Group 3 tumors are characterized by demethylated histone 3 lysine residue 4 (H3K4) (Figure 53). However, the SHH subgroup did have the most variable LSD1 expression range with outliers on both the low and high spectrum. For these reasons, deeper analysis of the SHH subgroup was required.

While there was no significant difference of overall LSD1 expression between patients with and without metastasis, the available patient data for these patients was not bountiful (Figure 54). Further, examination of LSD1 expression within patients with metastasis revealed that the majority of patients with metastatic MB had either moderate or high LSD1 expression (Figure 55).

In order to examine the potential of the tumors for epithelial to mesenchymal transition (EMT), we analyzed the expression levels of e-cadherin and vimentin (85). MB tumor dissemination and EMT is marked by an increase in expression of the filament protein, vimentin, along with the suppression of the cell adhesion molecule, e-cadherin. Analysis of patient samples with LSD1, saw a modest but significant positive correlation
with vimentin and negative correlation with e-cadherin (Figure 55). Together, these results suggest LSD1 may be involved in poor patient prognosis.

While these results are interesting, there are limitations to this dataset as it only contains transcript data and the importance of protein stabilization and activity is paramount to epigenetic activity of enzymes like LSD1. Due to this, analysis of stabilizers of LSD1 and REST (like USP7, Skp1, and BTRCP) can give more information on predicted protein availability and activity (71, (86))(Figure 29).

**Analysis of LSD1 target gene clustering in SHH MB**

To ask if LSD1 co-elevation with other genes or perturbation of LSD1 activity in SHH tumors may have more predictive value, we performed clustering analysis using a cohort of 91 LSD1 target genes, USP7-a gene controlling LSD1 and REST protein stability and 8 REST and metastasis related genes in the brain. Interestingly, analysis of clustering data revealed the existence of 6 clusters that aligned with the subtypes published previously by Cavalli et al (Figure 56) (7). The composition of the clusters was as follows: Cluster 1 -80% of SHH δ tumors, clusters 2 and 3 - 67% of SHH α tumors individually and together added up of 78% SHH α tumors, cluster 4 - 92% SHH β tumors, cluster 5 - 96% SHH Γ tumors, and cluster 6 was more undefined (Figure 57).

Interestingly, cluster 2 (subset of SHH α tumors) was characterized by highest increases in LSD1, REST, and USP7 expression of all clusters, while cluster 5 (SHH Γ tumors) exhibited the opposite pattern, with low expression of these genes (Figure 57). Cluster 3 (subset of SHH α tumors) exhibited LSD1 expression comparable to that in cluster 2, but more intermediate levels of REST, and USP7 expression remained
unchanged from cluster 1. Cluster 4 (SHH-β tumors) also had high expression of REST and LSD1 genes, but not USP7 (Figure 57). Tumors in clusters 5 (SHH-Γ tumors) and 6 (variable sub type distribution) exhibited elevated LSD1 expression, without co-elevation of REST or USP7 (Figure 57). These results indicate that co-elevation of REST and LSD1 transcript or increased stability of REST and LSD1 proteins could occur in subsets of SHH tumors. A significant correlation was observed between LSD1 and REST expression as well as LSD1 and USP7, \( r = 0.40; p<0.0001 \) and \( r = 0.38; p<0.0001 \) respectively, across these 4 clusters (Figure 57).

Further examination of patient outcomes between clusters 2/3 and 5 revealed a higher percentage of patients with metastasis in cluster 2/3 and 4 versus cluster 5 (Figure 58). Additional studies revealed a significant difference in Mantel-Cox analysis of survival curves of clusters 2-4 versus cluster 5 (Figure 58). Together, these results suggest that subsets of patients within the SHH α and β MB type have increased LSD1 and REST levels or activity and exhibit poor outcomes.
Figure 50. LSD1 transcript expression across MB subgroups. Transcript expression across subgroups of MB.
Figure 51. Survival curves of patients with low, moderate, or high LSD1 transcript levels. Overall survival is significant between conditions of low, moderate, and high LSD1 expression across all of MB patient samples (n=763). Gehan-Breslow-Wilcoxon (GBW) examination of early survival revealed a significant difference in early survival between low and high LSD1 patients (p<0.05). Mantel-Cox analysis of curves revealed that survival curves from moderate LSD1 and low LSD1 patients were significantly different (p < 0.05).
Figure 52. Survival curve of Group 3 patients shows significant relationship with LSD1 expression. Analysis of survival curves revealed significant differences in early and overall survival related to LSD1 transcript expression in Group 3 MB.
Figure 53. Survival curve of other MB subgroups. Kaplan-Meier survival curve differences between low, moderate, and high levels of LSD1 transcript were insignificant in A) WNT, B) Group 4, and C) SHH MB.
Figure 54. LSD1 expression in metastatic vs non-metastatic patients. Overall expression of LSD1 transcript between patients with and without metastasis A) across all MB samples and B) within MB subgroups. T-test analysis of differences in expression were insignificant in all cohorts.
Figure 55. LSD1 expression in metastatic patients. A) Analysis of patients with metastasis revealed that the majority of patients with metastasis had moderate to high transcript expression of LSD1. Further, B) LSD1 expression was positively correlated with Vimentin and C) negatively correlated with E-cadherin.
Figure 56. Clustering of SHH MB patients based on LSD1 target gene expression.

Clustering of MB patient samples was completed by a lab member, Shinji Maegawa, who used 91 genes previously established to be LSD1 target and REST and metastasis related genes in the brain. Clusters generated 6 clusters that loosely aligned with previously established SHH subtypes.
Figure 57. Clusters have distinct patterns of LSD1 and REST expression aligned with SHH MB subclassifications. A) Breakdown of SHH MB patient subtypes (alpha, beta, delta, gamma) per cluster (cluster 1 n=78; cluster 2 n=34; cluster 3 n=49; cluster 4 n=26; cluster 5 n=25; cluster 6 n=11). B) Box plots of LSD1 expression. Clusters 2, 3, and 4 were characterized by high levels of LSD1 and cluster 5 had significantly lower levels of LSD1 (*p<0.05; ****p<0.0001). Cluster 1 had significantly lower LSD1 expression than cluster 2. C) Box plot of REST expression. Clusters 2, 3, and 4 were characterized by high levels of REST and clusters 1 and 5 had significantly lower levels of REST (***p<0.001; ****p<0.0001). D) Box plot of USP7 expression. Cluster 2, but not 3 or 4, was characterized by high levels of USP7 and cluster 5 had significantly lower levels of USP7 (*p<0.05; **p<0.01; ***p<0.001). E) The expression of REST and LSD1 was correlated across 4 clusters (r = 0.40, p<0.0001). F) The expression of LSD1 and USP7 was correlated across 4 clusters (r = 0.38, p<0.0001).
Figure 58. Clusters 2, 3, and 4 have different patient outcomes from cluster 5. A) While clusters 2, 3, and 4 are both characterized by high LSD1 and REST, cluster 2 had a trend towards the highest amounts of REST and a higher levels or post-transcriptional stabilizer USP7. B) Analysis of patient outcomes revealed that patients from cluster 2/3 and 4 had a 6-fold and 4-fold increased incidence of metastasis. Kaplan-Meier survival curves from both Clusters 2, 3, and 4 were significantly lower than that of cluster 5 (p<0.01).
5.2 *In vitro* experiments

Genetic ablation of LSD1 expression or activity reduces MB cell viability.

To examine the requirement of LSD1 for MB cell growth, we used an LSD1-targeted shRNA in three SHH MB cell lines, UW228, UW426, and Daoy. LSD1 knockdown significantly reduced cell viability in both cell lines (Figure 59, Figure 60, Figure 61). Further, cell growth over time was also blocked by knocked down LSD1. At the molecular level, REST elevation increased LSD1 protein levels in both Daoy-REST and Uw288-REST cells (Figure 61). These results indicate that both the presence and activity of LSD1 is required for MB cell growth and that its repression can block the cell growth advantage of REST-expression in MB tumor cells.

The effect of LSD1 knockdown in the context of REST-overactivation was examined, as REST has been shown to protect LSD1 from degradation by maintaining the enzyme in complex (87). These results have implications for treating REST-high MB cells. Treatment of Daoy-REST cells with LSD1 knockdown also provided significant reduction in viability (Figure 61).

Irreversible inhibition of LSD1 has no effect on cell growth.

As we were able to demonstrate that both LSD1 function and activity could affect cell viability, we next evaluated a panel of irreversible LSD1 inhibitors on cell growth. However, within the MTT assay paradigm, we found that none of the irreversible inhibitors (tranylcypromine, GSKLSD1, and GSK2789552) had any effect on SHH MB cell lines UW228, UW426, and Daoy cells up to a 100 uM dose (Figure 62). Irreversible inhibition of LSD1 had no effect on UW228 or Daoy cells with elevated REST expression (Figure
A full cytotoxicity curve could only be capitulated with a dose range up to 1mM (data not shown).

**Irreversible inhibition of LSD1 reduces cell migration.**

Although the effect of the drug had no discernible effect on cell growth in MTT assays, previous study had found that LSD1 blockade could reduce the migration of MB cells. Using, scratch wound healing assays, we examined the effect of LSD1 inhibition on migration in normal and high-REST contexts. Irreversible inhibition reduced migration of UW426, UW228, and Daoy cells (Figure 64). While, UW228 cells did not migrate significantly in a 24 hour timeframe, UW228 cells with elevated REST expression resulted in a two-fold increase in cell migration (Figure 65). A similar two-fold change in migration was seen between Daoy and Daoy-REST cells (Figure 66). This migration advantage was blocked by LSD1 inhibition in a dose dependent manner using the irreversible inhibitor GSKLSD1. Daoy cells migrated more than UW228 cells within a faster time range of 18 hours. Further, LSD1 inhibition as low as 5 uM GSKLSD1 significantly reduced cell migration of Daoy cells (Figure 66). Daoy-REST cells migrated the most, with a two-fold increase compared to their Daoy counterparts (Figure 66). Interestingly, these cells also displayed a resistance to LSD1 inhibition at the lower dose, requiring a dose of 75 uM to significantly reduce migration.

We also examined transwell migration of Daoy and Daoy-REST cells to fetal bovine serum (FBS). Irreversible LSD1 inhibition at 75 uM was sufficient to significantly reduce cell migration over time in both Daoy and Daoy-REST cells (Figure 67). Together with the cell viability data, these results suggest that irreversible LSD1 inhibition may have a specific functional effect on MB cell migration.
Figure 59. LSD1 knockdown or catalytically inactive mutant of LSD1 decreases viability of UW228 SHH MB cell line. UW228 cell viability was reduced by genetic knockdown and catalytic inactive mutant of LSD1 (n=3; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).
Figure 60. LSD1 knockdown or catalytically inactive mutant of LSD1 decreases viability of UW426 SHH MB cell line. UW426 cell viability was reduced by genetic knockdown and catalytic inactive mutant of LSD1 (n=3; *p<0.05).
Cell Viability Over Time

Daoy
shNC
shLSD1

Daoy-REST
shNC
shLSD1

Cell Viability Over Time

Time (hours)
% cell viability

0 24 48 72
0 50 100

Daoy shNC
Daoy shLSD1

DR shNC
DR shLSD1

B

Daoy
Daoy-REST
UW228
UW228-REST

REST
LSD1
Actin
Figure 61. LSD1 knockdown or catalytically inactive mutant of LSD1 decreases viability of Daoy and Daoy-REST SHH MB cell line. A) Daoy and Daoy-REST cell viability was reduced by genetic knockdown and catalytic inactive mutant of LSD1 (n=3; p<0.05). B) Protein levels of REST and LSD1 were examined by western blot in isogenic cell pairs of UW228 and Daoy.
Figure 62. MTT assay with irreversible inhibitors of LSD1 in SHH MB cell lines. MTT assay of UW228, UW426, and Daoy cells were completed with three irreversible LSD1 inhibitors A) Tranylcypromine and Tranylcypromine derivatives: B) GSK2789552 and C) GSKLSD-1. Dose-response curves could not be generated in any cell lines or with any of the three drugs up to 100 μM dosage.
Figure 63. MTT assay with irreversible inhibitors of LSD1 in isogenic REST-transgene SHH MB cell line pairs. MTT assay of isogenic cell pairs A) Daoy/Daoy-REST and B) UW288/UW228-REST were completed with three irreversible LSD1 inhibitors (Tranylcypromine and Tranylcypromine derivatives: GSKLSD1 and GSK2879552). Dose-response curves could not be generated in any cell lines or with any of the three drugs up to 100 uM dosage.
Figure 64. Irreversible LSD1 inhibition reduces wound healing migration in SHH MB cell lines. Scratch wound healing assay was performed to examine the effect of LSD1 inhibition on migration using UW228, UW426, and Daoy cells. A panel of irreversible LSD1 inhibitors were used: A) Tranylcypromine and tranylcypromine derivatives; B) GSK2789552 and C) GSKLSD1. A low dose of 5 uM and a higher dose of 75 uM were used, neither which had any effect on cell growth in MTT. In most cases, a low dose of 5 uM was enough to reduce migration. (n=3; *p<0.05 **p<0.01).
Figure 65. GSKLSD1 reduces wound healing migration of UW228 and UW228-REST cells. Scratch wound healing assay was performed to examine the effect of LSD1 inhibition on migration of isogenic cell pair UW228 and UW228-REST. UW228 cells did not migrate much over a 24 hour period. UW228-REST cells migrated two-fold and a dose of 5 uM was sufficient to reduce migration (n=3; *p<0.05 **p<0.01).
Figure 66. GSKLSD1 reduces wound healing migration of Daoy and Daoy-REST cells. Scratch wound healing assay was performed to examine the effect of LSD1 inhibition on migration of isogenic cell pair Daoy and Daoy-REST. A dose of 5 uM was sufficient to reduce migration in Daoy cells. However, Daoy-REST cells migrated two-fold compared to Daoy and required 75 uM to significantly reduce migration (n=3; *p<0.05).
Figure 67. Irreversible LSD1 inhibition reduces IncuCyte transwell migration in Daoy and Daoy-REST cell lines. Time-coursed transwell migration assay was performed to examine the effect of LSD1 inhibition on migration of isogenic cell pair Daoy and Daoy-REST using the IncucyteZOOM system. Administration of 75 uM of GSKLSD1 flattened the slope of cell migration of both Daoy and Daoy-REST cells.
5.3 Analysis of RNA-sequencing data to determine targets of REST and LSD1

RNA-sequencing data demonstrates that irreversible inhibition of LSD1 functionally blocks migration machinery and reduces REST-dependent increase of HIF1A.

In order to obtain a better understanding of the molecular mechanism behind the peculiar specificity of LSD1 inhibition on cell migration, we treated Daoy cells with 75 uM of GSKLSD1 and performed RNA-sequencing. RNA-sequencing analysis revealed 451 significantly altered genes with 200 being upregulated and 251 being repressed by LSD1 inhibition (Figure 68). Functional analysis of these genes revealed several significant and relevant functions to cancer. These functions included cancer, cell death, cell adhesion, and migration. Further analysis of these cell migration and adhesion function illuminated several molecules of interest including previously established LSD1 target bone morphogenetic protein 2 (BMP2) and HIF1A (Figure 69). However, previous report had found that LSD1 knockdown restored BMP2 expression in MB cells. Interestingly, we found that while knockdown did indeed increase BMP2 expression, GSKLSD1 inhibition of LSD1 had the opposite effect (Figure 70). Although analysis of patient data examining BMP2 reinforced previous findings, showing that BMP2 and LSD1 are indeed negatively correlated. Although BMP2 was not increased with GSKLSD1 inhibition, REST related expression of HIF1A was reduced (Figure 71). HIF1A has been linked to both heightened REST and monoamine oxidase (MAO) inhibitors, such as Tranylcypromine (90). While these results may suggest that off target effects may be occurring with the use of tranylcypromine-derivative, GSKLSD1, they also show a beneficial effect of these
inhibitors on migration. Further, GSKLSD1, and other analogs of tranylcypromine, have an N-amine substituent increasing its specificity for LSD1. Further previous studies have demonstrated a role for LSD1 in regulating HIF1A expression and protein stability. While REST normally acts as a repressor at the gene promoter of HIF1A, among other targets, the role of cytoplasmic REST and LSD1 in the context of proteosomal stabilization or nuclear localization and function of REST has yet to be explored (91, 92). Additionally, increased REST expression has been shown to influence REST expression to a more cytoplasmic localization that can increase the stability of proteins (93). In line with these postulations, HIF1A was positively correlated with LSD1 and REST and patient samples (Figure 72).

However, it is important to note that in these experiments LSD1 inhibition nor knockdown in Daoy and Daoy-REST cells resulted in global dimethylation changes at H3K4 or H3K9 residues as measured by western blot of histones purified by acid extraction (Figure 73).

REST has been previously linked to HIF1A expression and activity. However, these results are the first to show that LSD1 is involved in REST-dependent HIF1A expression especially in a positive manner (Figure 71, Figure 72). Further, while GSKLSD1 had no effect on BMP2, GSKLSD1 inhibition did reduce HIF1A levels which were positively correlated with REST expression in patient samples. These findings provide a novel target for REST and LSD1 coactivity as a stabilizer of HIF1A expression. Although, off-target effects of GSKLSD-1 cannot be excluded and these effects warrant further investigation. More studies are necessary to examine subgroup-specific changes in LSD1 target genes.
Figure 68. RNA-sequencing and functional analysis of irreversible LSD1 inhibitor treated Daoy cells. Daoy cells were treated with 75 uM of GSKLSD1 and submitted for RNA-sequencing. A) Volcano plot shows that a subset of genes were significantly altered (red dots; p<0.05). B) IPA analysis of significantly altered functions revealed several functions of interest, including cancer (not pictured here), cellular death, and cell movement (p<0.01).
Figure 69. Heatmap identifies genes involved in cell adhesion and migration function altered by irreversible LSD1 inhibition. Genes from cellular adhesion and migration revealed HIF1A, but not BMP2, as a target of LSD1 inhibition.
Figure 70. BMP2 expression is upregulated by LSD1 knockdown but not irreversible inhibition.  A) qPCR of Daoy and Daoy-REST cells treated with shLSD1 or GSKLSD1 revealed that BMP2 was differentially regulated (n=3; *p<0.05). B) Analysis of patient data revealed that LSD1 and BMP2 were indeed correlated and related to clusters 2, 3, 4, and 5 in a REST and LSD1 dependent manner (****p<0.0001).
Figure 7. Irreversible inhibition of LSD1 reduces HIF1A expression in the context of endogenous and elevated REST levels. qPCR of isogenic cell pairs of Daoy and UW228 cells treated with GSKLSD1 revealed that HIF1A was reduced (n=3; **p<0.01; ****p<0.0001).
Figure 72. HIF1A is correlated with high REST and LSD1 levels in patient samples between clusters 2 and 5. A) Analysis of patient data revealed that LSD1 and HIF1A were correlated along with REST expression in clusters 2, 3, and 5 (***p<0.001). B) Interestingly, cluster 3 trended differently than cluster 2.
Figure 73. Histone extraction reveals no change in global dimethylation of H3K4 or H3K9 in response to inhibition or knockdown. Global changes were not detectable in response to LSD1 targeting in Daoy or Daoy-REST cells.
In brief, the combination of these results highlight the potential of targeting LSD1 function in the context of high REST. LSD1 is elevated in MB samples is related to lower survival in patient samples. Further, the coactivity of LSD1 and REST seems to be specifically relevant in subsets of patients clustered by LSD1 target genes (clusters 2 and 3). These clusters mostly aligned with SHH alpha subtypes. When contrasted with patients clustered with lowered levels of LSD1 and REST (cluster 5), significant differences in overall survival were observed. In *in vitro* experiments, LSD1 knockdown and genetic inactivation reduced SHH MB cell line viability over time. Further, REST overexpression in UW228 and Daoy cells (UW228-REST, DAOY-REST) resulted in increased LSD1 protein levels and increased cell growth in Daoy-REST cells. These cell growth advantages were blocked by LSD1 knockdown. Interestingly, use of irreversible inhibitors derived from the MAO inhibitor, Tranylcypromine, had no effect on cell growth on SHH MBs with or without high REST expression. However, within the same dose range, irreversible LSD1 inhibition reduced cell migration as measured by scratch wound healing assay. Transwell migration of isogenic cell pair Daoy and Daoy-REST revealed that LSD1 inhibition significantly reduced migration as well. Daoy cells with endogenous levels of REST treated with LSD1 inhibitor were submitted for RNA-sequencing to identify expression changes occurring with inhibition. Functional analysis of RNA-sequencing data revealed several significantly altered functions including cancer and cellular movement. Closer analysis of gene expression changes revealed a previously established target, BMP2, with an opposite expression pattern of expression from genetic knockdown, that was confirmed with qPCR. Another LSD1 target, HIF1A, was also a candidate from the RNA-sequencing. HIF1A transcript expression was verified to reduce
with LSD1 inhibition in the context of both low and high REST. Analysis of the patient data revealed a significant positive correlation of REST and HIF1A along with LSD1. These preliminary results are the first to suggest that LSD1 may positively regulate HIF1A in the context of REST.
Chapter 6:

Discussion and Future Directions
The need for targeted therapies in pediatric MB is dire. While subsets of patients have good prognosis and an 80% five-year event free survival, other subsets of patients experience heightened rates of metastasis and recurrence (2, 6). For these patients, standard care does little to ease tumor progression and aggressive treatments can severely reduce quality of life without guarantee of efficacy. Even when successful, radiation therapy increases the risk of neurocognitive defects when used on younger patients. Outside of these options, hospice care is the only remaining comfort that the clinic can offer. Personalized medicine will pave the way to target tumor cells without affecting normal tissues as much. While radiation and chemotherapy are still our best tools for fighting tumor growth, it is the hope of the cancer field that, while we can initially work in conjunction with these tools, we can reduce our use of these aggressive and nonspecific therapies.

In order to develop and utilize targeted therapies, we first have to understand the underlying biological mechanisms of tumor progression and development. Understanding the rational for molecular targeting allows for strategies such as synthetic lethality to be successful. Synthetic lethality is a principal discovered in drosophila experiments demonstrating that a given organism will die when the expression of two genes is simultaneously blocked but that blockade of either gene in isolation has no effect on organismal viability (94, 95).

The principal of synthetic lethality gained traction in the cancer research community in the early 21st century. Due to the existence of several redundant cellular mechanisms regulating homeostasis, metabolism, and cell cycle progression, there are many molecular hurdles that a normal cell must pass to transform into a tumor cell. In
fact, for many cells there are several proteins and pathways with redundant functions. This acts as a fail-safe that allows cells and tissues to overcome injuries over time. This principle is exactly why cancer is largely viewed as a disease of aging. Over time, our aging tissues accumulate insults and injuries that contribute to the formation of mutations. Genetic predispositions, meaning having inherited mutations in oncogenes or tumor suppressors, contribute to the ever-growing checklist of “hits” that your cells can take. In the same way, risk factors that directly injure your tissues or increase the generation of harmful reactive oxygen species can also increase the likelihood of mutations. When these mutations accumulate in such a way that cell growth goes unchecked, cells can grow out of control and become tumors. Further, tumorigenic cells are often also programmed to evade immune system checks and apoptosis. Because tumor cells are hallmarked by genetic instability and loss-of-function mutations in tumor suppressor genes, there are many cases where tumors become “addicted” to the expression of certain genes that allow them to proliferate even though they are genetically handicapped in many ways. This is the primary reason that tumor cells can be prime candidates for synthetic lethality. Let’s say that genes A and B are two redundantly-similar genes that promote survival, normal cells are able to utilize both gene A and gene B pathways. Due to the accumulation of mutations over time, a tumor cell lost gene A and it is now dependent on gene B for survival. Now a clinician administers a drug that blocks the function of gene B. Normal cells will switch over to gene A to survive while the tumor cell will die. This is synthetic lethality. This landmark cancer therapeutic strategy was first put to use in breast cancer with mutations in BRCA 1/2 in 2005 (96). BRCA 1/2 are molecules important for repairing double-stranded DNA breaks. Loss-of-function mutations in BRCA
1/2 predispose individuals to breast cancer. Another molecule involved in repairing single-standed breaks is PARP1. When tumor cells with BRCA 1/2 mutations are targeted with PARP1 inhibitors, they are unable to overcome the load of DNA-damage and perish. This principal is now being applied to several different types of cancers, including MB. However, a greater understanding of molecular mechanisms of tumorigenesis is required. Synthetic lethality is a quintessential example of how heightened mechanistic understanding allows for successful targeted therapeutics. The goal of my work has been to uncover targetable mechanisms for MB metastasis and progression.

Our lab and others has demonstrated that REST is a driver of MB progression and leptomeningeal dissemination. REST is a canonical regulator of brain development. Its expression is downregulated during neuronal lineage specification, which results in derepression of expression of its target neuronal differentiation genes (54-57). REST levels are elevated in the pediatric brain tumor MB, and are associated with a poor overall and event free survival (47). However, the mechanisms of the role of REST in these processes were under investigated. While it was demonstrated that REST alone cannot generate tumors, we found that REST expression could alter the molecular properties of granule neuron progenitors in the cerebellum. Further, these cells with upregulated REST were more sensitive to chemokinesis and had issues organizing correctly in the cerebellum. In the context of SHH MB, an increase in REST expression in GNPs in the context of constitutive activation of SHH signaling drove an aggressive disease course associated with increased tumor penetrance, decreased latency, and leptomeningeal dissemination of MB cells. Fortunately, these results have also provided mechanistic insights into normal postnatal cerebellar development and how it is co-opted during tumorigenesis, which is
consistent with the notion that MB is a disease of normal development gone awry (21, 24, 31, 32).

The cerebellum is the most frequent site of MB growth and is unique for many reasons. Its complex perinatal development, which can last up to two years after birth, makes it vulnerable to insult and malformation (21-23). Successful cerebellar development requires a multitude of coordinated cellular and molecular processes, the study of which has been pivotal to understanding MB genesis. An aspect that is of particular relevance to this study involves the developmentally regulated expansion, migration, and differentiation of GNPs. Our work described here is the first to demonstrate that deregulated expression of REST perturbs the directed migration of GNPs from the EGL to the IGL during normal postnatal cerebellar development. Probing the RNA-sequencing data with the observation that many of the migration defects take place in the progenitor-populated EGL and at the meningeal barrier led us to more carefully investigate a hypothetical connection between REST elevation and changes in CXCR4 signaling. The CXCR4 pathway is vital to proper guidance of GNPs in the EGL before they commit to radial migration to their final destination in the IGL (39). Genetic loss of CXCR4 or SDF1 causes premature GNP migration and foliation defects (96, 97). The meningeal epithelium is known to secrete SDF1, which is a ligand for the chemokine receptor CXCR4 present on GNPs. This interaction may spatially constrain GNPs within the outer EGL, where they undergo a rapid proliferative phase and expansion coincident with tangential migration (39, 98, 99). While the effects of CXCR4 and SDF1 knockdowns on cerebellar development have been extensively studied, the consequences of CXCR4 elevation is still unknown. In our mouse model, REST represses BDNF expression, which
has been demonstrated to negatively regulate CXCR4 activity. These molecular events may favor a state in which cells are restrained within the EGL space, and possibly even facilitate tangential migration.

REST elevation also upregulates ERK levels at the protein level, suggesting affected downstream signaling. While it is unlikely that REST regulates ERK at the transcriptional level, REST has been demonstrated to be capable of inhibiting ubiquitinases which may be a means of protein stabilization (46). This idea may be strengthened by our observation that while SDF1 transcript was down in our RNA-sequencing data, the protein levels were increased. This is an observation that we see often in our REST-expressing cells, somewhat surprisingly as REST is a canonical repressor. However, repression of players in protein degradation pathways or differential functions of REST in the nuclear/cytoplasmic compartments could be an interesting explanation for this phenomena. In line with this, previous preliminary work has demonstrated that high levels of REST result in increased cytoplasmic expression that is capable of stabilizing luciferase-tagged proteins \textit{in vitro} (100). Further studies are needed to elucidate the role of REST in the protein degradation pathway.

An ongoing study under review has shown that REST also regulates proliferation of SHH MBs, a subgroup of which arise from GNPs. The proliferative phase undergone by GNPs in the EGL is followed by the onset of neurogenesis, marked by a decline in REST levels, derepression of REST target neuronal differentiation genes, and is accompanied by radial migration across the molecular layer to its final destination in the IGL. Radial migration of differentiating neurons requires radial glial processes as a scaffold (98). The number and organization of these cells seem to be abnormal in the
cerebellum of REST\textsuperscript{TG} mice. The molecular reasons underlying this phenomenon is not clear, but it would be reasonable to speculate that cell-cell communication and dependency may be bi-directional between neurons and radial glia (10, 13, 22, 101, 102). Although, the scope of this work does not include neuron-glia interactions, the importance of these interactions is an apparent future direction of this work. Further, other work on our lab has focused on understanding how REST and SHH signaling cooperate to promote tumorigenesis. As REST is being expressed in the same GNPs that are responding the SHH secreted by the purkinje cells, the opportunity for cooperativity between the two pathways is present. In the scope of mechanisms of SHH signaling regulation, so much is still unknown about interactions after Smo activation that stabilize Gli and allow nuclear translocation. Current postulations include differential phosphorylation of Gli into active 1 and 2 states that translocate. Nothing is known about what happens to phosphorylation regulators that direct Gli for ubiquitnation and NH2 terminal truncation into Gli 3. A paper currently being published in our lab speculates on the role of REST in Gli stabilization and transcription along with a role for REST in transcriptional repression of the PTCH1 receptor.

CXCR4/SDF1 engagement can result in activation of a number of downstream signaling cascades, including ERK, AKT, and calcium signaling (39, 42). REST and CXCR4 seem to be expressed in MB in a cooperative manner and while we saw a decrease in REST-dependent migration, the effect of CXCR4 antagonism by AMD3100 was modest. This may be due to additional mechanisms of increased migration by the tumor. Additionally, the patient data we analyzed suggests a strong dependence of these effects on SHH signaling and imply a subtype-specific effect. The cooperativity of REST
and CXCR4 seem to peak in SHH-beta and SHH-delta subgroups. Further studies determining the subgroup of commercially available cell lines, like UW228 and Daoy, may allow more precise experiments on these molecular interactions. Our results described here along with previous studies in other labs highlight the importance of understanding how molecular signaling in different subgroups contribute to individual differences in tumor genesis and progression (7, 8, 103, 104).

The work of chapters 3 and 4 of this dissertation is the first to link REST to the regulation of GNP migration and to the regulation of CXCR4/SDF1 signaling, providing the groundwork to explore the potential of targeted therapeutic options for a subset of SHH patients experiencing tumor dissemination with increased REST expression. We also examined the expression levels of known REST target brain-derived neurotrophic factor (BDNF) in the tumor environment as BDNF has the potential to inhibit CXCR4 activity. We demonstrate that REST elevation can reduce BDNF and increase CXCR4 and SDF1 expression, molecules with established roles in both normal cerebellar development and in MB. These studies opens the door to exploring CXCR4 blockade as a therapeutic option for a subset of patients. Our findings not only provide an additional facet to REST’s contribution to SHH-specific MB development, but also highlight the potential of disrupting the highly regulated normal postnatal cerebellar development in tumorigenesis.

Other studies have shown that increased signaling through AKT, outside of CXCR4 activation, can also influence migration (105-108). It may be necessary to use combination therapy to target multiple pathways including CXCR4 and AKT in order to completely block migration. Altogether, REST is an attractive molecular target for
pediatric brain cancers including MB. Recently, labs are making more selective inhibitors for REST complex activity for use in these contexts (74). Outside of these intriguing small molecule inhibitors, other drugs are already on the market for REST complex partners with epigenetic activity. Some that our lab have worked with include REST-coREST partners LSD1, a lysine-specific demethylase which has been implicated in MB cell growth and the methyltransferase G9a, which has been demonstrated to play a role in the cell growth and proliferation of MB human cell lines (46, 73, 74, 78, 79).

The work completed with the aim of targeting LSD1 in the context of REST has demonstrated some interesting results. The work completed has demonstrated that LSD1 is necessary for cell growth of SHH MB, both in the context of high and low REST. However, low-potency irreversible inhibition of LSD1 reduces cell migration of SHH MB in a REST-dependent manner. This inhibition is paired with reduced HIF1A expression. Interesting results from this aim raises questions about specific functions of LSD1 and also highlighted the importance of specific targeting strategies against LSD1. The fact that genetic knockdown of LSD1 resulted in a different expression pattern than irreversible inhibition warrants further investigation into the mechanism of action of different classes of inhibitors. Some clinical observations have stated that inhibitors only partially targeting enzyme activity can not only have off-target effects but can sometime result in unintended compensatory activity.

Along these lines, a new class of inhibitors against LSD1 is being explored. With a different mechanism of action, these new inhibitors are considered to be reversible inhibitors. Tranylcypromine, GSK2789552, GSKLSD1 all irreversible, tranylcypromine derivatives that act on FAD enzyme activity to inhibit oxidative process necessary for
demethylation (109). Amine-containing N-substituents added introduced to tranicyclepromine moiety increased inhibitory activity and lent the drugs with more specificity to LSD1. New reversible inhibitors of LSD1 have been developed by Salarius pharmaceuticals and include SP2509 and SP2557. SP2577 is a derivative of SP2509, a reversible LSD1 inhibitor that acts by preventing LSD1 binding to cofactors like coREST (110). Sp2577 is the more potent that commercially available inhibitors and its mode of action is different than the three used previously. Although the irreversible, tranicyclepromine-derived drugs were stouted to be LSD1 specific due to not having an effect on cell growth of over 100 other cell lines (111), other reaction phenotypes outside of cell growth were still seen by treatment in studies done by our lab and others. Further, preliminary results have shown that genetic knockdown, inactivity, and indeed use of the reversible inhibitor sp2577 proved to inhibit SHH MB cell growth and viability (data not shown).

Another point of interest is that REST and CXCR4 cooperativity appears to occur most relevantly in SHH β subgroup of MB. On the other hand, LSD1 activity appears to be the most relevant in the SHH α subgroup. While SHH β subgroup has the highest amount of metastasis, the age range of patients is on the lower end with most patients being 0-3 years of age. The SHH α patients on the other hand are still pediatric but take place in older children. These patients have the second highest incidence of metastasis. As SHH β patients still have developing cerebella (with postnatal cerebellar lamination continuing until the age of 2 years), it is encouraging that our results suggest that yet another developmental pathway (CXCR4) cooperates with REST in a manner that promotes tumorigenesis and dissemination. In the older pediatric patients of the SHH α
subgroup, we see a different mechanism independent of this developmental CXCR4 signaling. Instead these patients seem to be more sensitive to the increased levels of epigenetic histone modifier, LSD1. That REST cooperates with different pathways depending on the availability of these programs suggests that elevated REST could be coordinating with several programs to drive MB tumorigenesis and dissemination.
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Vita

Keri Callegari was born in Houston, Texas as the daughter of Kimberly Hoppe and Andres Callegari. After completing her work at Kingwood High School (Kingwood, Texas) in 2009, she entered Texas A&M University in College Station, Texas. She received the degree of Bachelor of Science with a major in Psychology and a minor in Neuroscience from Texas A&M in May, 2013. In August of 2013 she entered The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences.

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