Regulation of Cerebellar Development and Tumorigenesis by CXCR4 and by Aurora and Polo-Like Kinases

by

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David Kirsch

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

2013
ABSTRACT

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Abstract

During development, the precise regulation of proliferation, migration, and differentiation is required to establish proper organ structure/function and prevent deregulation that can lead to disease, such as cancer. Improved understanding of signals that regulate these processes is necessary to gain insight into the mechanisms of organ development and to identify strategies for treating the consequences of deregulation. In the cerebellum, factors that regulate these processes remain incompletely understood. Our studies have focused on the migration of cerebellar granule neuron progenitors (GNPs) and the contribution of the SDF-1/CXCR4 axis to postnatal development. Using conditional knockout mice, we show that deletion of CXCR4 causes premature migration of a subset of GNPs throughout development that are capable of proliferation outside of their normal mitogenic niche. Loss of CXCR4 also causes a reduction in activity of the Sonic hedgehog (SHH) pathway (the primary mitogen for GNPs) but does not affect GNP proliferation, differentiation, or capacity for tumor formation. Our data suggest that SDF-1/CXCR4 signaling is necessary for proper migration of GNPs throughout cerebellar development.

In addition to understanding the signals that regulate normal development, the identification of vulnerabilities of established tumors is also necessary to improve cancer treatment. One strategy to achieve this involves targeting cells that are critical for...
maintaining tumor growth, known as tumor-propagating cells (TPCs). In the context of the cerebellar tumor medulloblastoma (MB), we previously identified TPCs in tumors from patched mutant mice that express the carbohydrate antigen CD15. Here, we employed multiple approaches to target these cells, including biochemical identification of molecules that carry the CD15 carbohydrate epitope and immunotoxin-mediated targeting of CD15-expressing cells. Unfortunately, these strategies were ultimately unsuccessful, but an alternative approach that recognized a vulnerability of CD15+ cells was identified. We show that CD15+ cells express elevated levels of genes associated with the G2/M phases of the cell cycle, progress more rapidly through the cell cycle than CD15– cells, and contain an increased proportion of cells in G2/M. Inhibitors of Aurora and Polo-like kinases, key regulators of G2/M, induce cell cycle arrest, apoptosis and enhanced sensitivity to conventional chemotherapy, and treatment of tumor-bearing mice with these agents significantly inhibits tumor growth. Importantly, cells from patient-derived MB xenografts are also sensitive to Aurora and Polo-like kinase inhibitors. Our findings suggest that targeting G2/M regulators may represent a novel approach for the treatment of human MB.
Contents

Abstract ................................................................................................................................................iv
List of Figures ........................................................................................................................................xi
Abbreviations .........................................................................................................................................xiii
Acknowledgements ..............................................................................................................................xv
1. Introduction ........................................................................................................................................1
   1.1 Cerebellar Development ................................................................................................................1
       1.1.1 Tangential migration of GNPs ..............................................................................................3
       1.1.2 Proliferation of GNPs ...........................................................................................................4
       1.1.3 Differentiation and radial migration of GNPs ........................................................................6
       1.1.4 SDF-1/CXCR4 signaling .......................................................................................................9
           1.1.4.1 SDF-1/CXCR4 in the central nervous system ..........................................................11
           1.1.4.2 Role of SDF-1/CXCR4 in postnatal cerebellar development ..................................12
       1.2 Tumorigenesis ..........................................................................................................................13
           1.2.1 Medulloblastoma subtypes .............................................................................................14
               1.2.1.1 SHH-associated subtype of medulloblastoma .........................................................15
               1.2.1.2 Mouse models for SHH-associated subtype .........................................................16
           1.2.2 Tumor-propagating cells .................................................................................................19
               1.2.2.1 Tumor-propagating cells in brain tumors ...............................................................20
               1.2.2.2 Tumor-propagating cells in SHH-associated medulloblastoma ............................22
           1.2.3 Targeting CD15+ tumor-propagating cells .....................................................................23
2. CXCR4 is required for appropriate radial migration of cerebellar granule neuron progenitors ................................................................. 26

2.1 Introduction ........................................................................................................... 26

2.2 Methods ................................................................................................................. 29
  2.2.1 Mice .................................................................................................................. 29
  2.2.2 Tissue collection ............................................................................................... 30
  2.2.3 Immunostaining ............................................................................................... 31
  2.2.4 In situ hybridization ....................................................................................... 31
  2.2.5 Isolation of GNPs ............................................................................................ 32
  2.2.6 Real-time PCR .................................................................................................. 33
  2.2.7 Proliferation assays ........................................................................................... 33
  2.2.8 In vitro response to Shh ................................................................................... 34
  2.2.9 Tumor formation ............................................................................................... 35

2.3 Results .................................................................................................................. 35
  2.3.1 CXCR4 deletion causes premature GNP migration away from the EGL into the IGL and white matter ................................................. 35
  2.3.2 Ectopic cell clusters are granule neuron progenitors ........................................ 38
  2.3.3 Premature migration of GNPs causes disruptions in Bergmann glial and Purkinje layer organization .......................................................... 39
  2.3.4 CXCR4-deficient GNPs exhibit reduced Shh target gene expression, but proliferate normally ................................................................. 42
  2.3.5 Ectopic GNPs differentiate appropriately ........................................................ 46
  2.3.6 CXCR4 is not required after postnatal day 4 for appropriate GNP migration. 48
2.3.7 Loss of CXCR4 does not impair tumor formation .................................................. 50
2.4 Discussion ...................................................................................................................... 52

3. Biochemical Identification of CD15-associated molecules and Immunological Targeting of CD15+ Tumor-Propagating Cells ................................................................. 60

3.1 Introduction .................................................................................................................. 60
3.2 Methods ........................................................................................................................ 63

3.2.1 Methods for biochemical identification of CD15 carrier molecule ......................... 63

3.2.1.1 Cell lines and mice .............................................................................................. 63
3.2.1.2 Isolation of patched mutant tumor cells .............................................................. 63
3.2.1.3 Generation of anti-CD15-agarose ...................................................................... 64
3.2.1.4 Immunoprecipitation and Western blot .............................................................. 64
3.2.1.5 Cross-linking of antibody to cells ....................................................................... 65
3.2.1.6 Removal of CD15-associated proteins and lipids from cell surface ............. 66
3.2.1.7 Flow cytometry to assess CD15 expression ....................................................... 66

3.2.2 Methods for immunotoxin-based targeting of CD15+ cells ................................. 66

3.2.2.1 Reagents ............................................................................................................. 66
3.2.2.2 Proliferation of cells cultured in the presence of immunotoxin ...................... 67

3.3 Results ........................................................................................................................ 67

3.3.1 Biochemical identification of CD15 carrier molecule ............................................. 67

3.3.1.1 Immunoprecipitation/Western blot approach can identify a CD15-associated molecule in F9 cells .......................................................... 67

3.3.1.2 Immunoprecipitation/Western blot approaches fail to identify a CD15-associated protein in patched mutant tumor cells ........................................ 69
3.3.1.3 CD15 may be associated with both protein and lipid molecules in patched mutant tumor cells .............................................................. 74

3.3.2 Immunotoxin-mediated targeting of CD15+ cells ........................................... 76

3.3.2.1 CD15 antibody plus Saporin-conjugated secondary antibody can effectively inhibit proliferation ................................................................. 76

3.3.2.2 Directly conjugated CD15-SAP immunotoxin specifically inhibits proliferation of F9 cells ........................................................................ 78

3.3.2.3 Both control IgM-SAP and CD15-SAP inhibit proliferation of patched mutant tumor cells ........................................................................ 79

3.4 Discussion ......................................................................................................... 82

4. Targeting Sonic Hedgehog-associated medulloblastoma through inhibition of Aurora and Polo-Like Kinases .................................................................. 89

4.1 Introduction .................................................................................................... 89

4.2 Materials and Methods .................................................................................. 91

4.2.1 Mice ........................................................................................................... 91

4.2.2 Human tumor isolation and propagation ................................................... 91

4.2.3 Chemicals .................................................................................................. 92

4.2.4 Tumor cell isolation and culture ................................................................. 92

4.2.5 Cell sorting ................................................................................................ 93

4.2.6 Real-time PCR .......................................................................................... 93

4.2.7 BrdU and cell cycle analysis ...................................................................... 94

4.2.8 Western blotting ...................................................................................... 95

4.2.9 Proliferation assays ................................................................................. 96

4.2.10 In vivo drug administration ..................................................................... 96
4.3 Results ........................................................................................................................................... 97

4.3.1 CD15+ cells display elevated expression of G2/M regulators ............................................. 97

4.3.2 CD15+ cells are enriched in G2/M ......................................................................................... 98

4.3.3 CD15+ cells progress more rapidly through the cell cycle than CD15- cells .... 99

4.3.4 Targeting G2/M regulators blocks progression through the cell cycle and inhibits proliferation .......................................................... 101

4.3.5 Plk inhibition cooperates with SHH antagonist and conventional chemotherapy ................................................................. 106

4.3.6 Inhibition of G2/M regulators blocks tumor growth in vivo .............................................. 110

4.3.7 Aurk and Plk inhibitors suppress growth of human SHH-associated MB ......... 114

4.4 Discussion ..................................................................................................................................... 116

5. Conclusions and perspectives .................................................................................................. 123

5.1 CXCR4 is required for appropriate migration in the cerebellum .......................... 124

5.2 Identification of CD15-associated carrier molecules and approaches for targeting CD15+ tumor-propagating cells .................................................................................. 129

5.3 Targeting CD15+ cells through inhibition of G2/M cell cycle regulators .......... 133

5.4 Concluding statement ............................................................................................................. 138

References ........................................................................................................................................... 140

Biography ........................................................................................................................................ 163
List of Figures

Figure 1.1: Modes of migration in the developing cerebellum..................................................3

Figure 2.1: Loss of CXCR4 results in the presence of ectopic cell clusters throughout cerebellar development...........................................................................................................37

Figure 3.1: Structure of the CD15 epitope. ................................................................................62

Figure 3.2: CD15 antibody can immunoprecipitate high molecular weight protein in F9 teratocarcinoma cells. ..................................................................................................................69

Figure 3.3: CD15 antibody does not immunoprecipitate an associated protein from patched mutant tumor cells.................................................................71

Figure 3.4: Cross-linking of CD15 antibody to CD15 epitope on cell surface does not enable identification of CD15-associated protein.................................................................73

Figure 3.5: CD15 epitope detection is affected by removal of proteins and lipids from the cell surface..........................................................................................................................75

Figure 3.6: Treatment of F9 cells or patched mutant tumor cells with CD15 primary antibody plus saporin-conjugated secondary antibody inhibits proliferation. ................77

Figure 3.7: Saporin-conjugated anti-CD15 antibody inhibits proliferation of F9 cells but not Ba/F3 cells............................................................................................................................79

Figure 3.8: patched mutant tumor cells are sensitive to control IgM-SAP. .........................81

Figure 4.1: CD15+ cells display elevated expression of Aurora and Polo-like kinases and an increased proportion of cells in G2/M .................................................................98

Figure 4.2: CD15+ cells progress more rapidly through the cell cycle than CD15- cells. ......................................................................................................................................................100

Figure 4.3: Aurk and Plk inhibitors block proliferation and cell cycle progression......103

Figure 4.4: Aurk and Plk inhibitors block proliferation of patched mutant...............105

Figure 4.5: Plk inhibitor cooperates with SHH antagonist and chemotherapeutic agents. .................................................................................................................................108
Figure 4.6: Aurk inhibitor cooperates with SHH antagonist and chemotherapeutic agents................................................................. 109

Figure 4.7: Plk inhibitor blocks the growth of patched tumors in vivo.......................... 112

Figure 4.8: Aurk inhibitor blocks tumor growth in vivo............................................. 113

Figure 4.9: Aurk and Plk inhibitors suppress proliferation of human SHH-associated medulloblastoma......................................................... 115
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>EGL</td>
<td>External granule layer</td>
</tr>
<tr>
<td>IGL</td>
<td>Internal granule layer</td>
</tr>
<tr>
<td>VZ</td>
<td>Ventricular zone</td>
</tr>
<tr>
<td>URL</td>
<td>Upper rhombic lip</td>
</tr>
<tr>
<td>GNP</td>
<td>Granule neuron progenitor</td>
</tr>
<tr>
<td>MB</td>
<td>Medulloblastoma</td>
</tr>
<tr>
<td>TPC</td>
<td>Tumor-propagating cell</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>Ptch1</td>
<td>Patched1</td>
</tr>
<tr>
<td>Smo</td>
<td>Smoothened</td>
</tr>
<tr>
<td>SuFu</td>
<td>Suppressor of Fused</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal-derived factor 1</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>Sema</td>
<td>Semaphorin</td>
</tr>
<tr>
<td>BLBP</td>
<td>Brain lipid binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>GABRA6</td>
<td>GABA receptor alpha 6</td>
</tr>
<tr>
<td>SSEA-1</td>
<td>Stage-specific embryonic antigen 1</td>
</tr>
<tr>
<td>AurkA</td>
<td>Aurora kinase A</td>
</tr>
<tr>
<td>AurkB</td>
<td>Aurora kinase B</td>
</tr>
<tr>
<td>Plk1</td>
<td>Polo-like kinase 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>SAP</td>
<td>Saporin</td>
</tr>
<tr>
<td>mZAP</td>
<td>Anti-IgM-Saporin antibody</td>
</tr>
<tr>
<td>^3H-Td</td>
<td>^3H-thymidine</td>
</tr>
<tr>
<td>IC_{50}</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>DSS</td>
<td>Disuccinimidylyl suberate</td>
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1. Introduction

The coordination of proliferation, migration, and differentiation is critical for the formation of the mature nervous system, and deregulation of these processes can contribute to the development of central nervous system (CNS) malignancies. Elucidation of the signals that regulate proliferation and migration in the CNS is necessary to improve our understanding of normal brain development and the processes that become deregulated in tumorigenesis.

1.1 Cerebellar Development

The developing cerebellum represents an ideal context in which to study the factors that regulate neuronal proliferation and migration. The cerebellum is composed of multiple cell types, including granule, Purkinje, Golgi, stellate and basket neurons as well as several classes of astrocytes and oligodendrocytes. Each of these cell types is derived from one of two germinal zones in the embryonic cerebellum: the ventricular zone (VZ) and the external granule layer (EGL) (Wang and Zoghbi 2001) (ten Donkelaar et al. 2003) (Hatten and Roussel 2011). The VZ consists of a layer of stem cells within the neuroepithelium around the roof of the fourth ventricle. The majority of these cells proliferate during embryogenesis and then differentiate and migrate radially toward the interior of the cerebellum, giving rise to the majority of cerebellar neuronal and glial cell types.
An additional subset of cells from the embryonic VZ moves laterally to a structure called the upper rhombic lip (URL). These cells begin to express the transcription factor Math1 (Atoh1) and commit to the granule neuron lineage (Wang and Zoghbi 2001) (ten Donkelaar et al. 2003) (Hatten and Roussel 2011). Development of granule neurons, the most abundant cell type in the cerebellum, is known to proceed through a relatively well-established sequence of proliferation, migration, and differentiation. After migrating past the URL, lineage-committed granule neuronal progenitors (GNPs) continue to migrate tangentially over the surface of the cerebellum and simultaneously undergo extensive proliferation, forming the layer of GNPs known as the EGL. In mice, the EGL expands throughout embryonic stages until one week after birth, then shrinks and disappears by 3 weeks after birth as GNPs ultimately undergo differentiation and radial migration to the interior of the cerebellum to form the internal granule layer (IGL) of mature granule neurons. The modes of migration within the developing cerebellum are depicted in Figure 1.1.
**Figure 1.1: Modes of migration in the developing cerebellum.**

**Figure 1.1.** Cells originating from the neuroepithelium of the ventricular zone migrate over the rhombic lip and begin to express the transcription factor Math1 to become GNPs. The GNPs then proliferate and migrate tangentially to cover the surface of the cerebellum, forming the external granule layer. Upon cell cycle exit, GNPs undergoing differentiation leave the EGL and migrate radially towards the interior of the cerebellum to their final position in the internal granule layer. Image modified from an E17.5 cerebellum stained with DAPI.

### 1.1.1 Tangential migration of GNPs

Within the rhombic lip, Math1-expressing GNPs begin to proliferate, acquire a unipolar morphology with a single process that projects away from the rhombic lip, and begin to migrate anteriorly from the rhombic lip to spread over the surface of the
cerebellum and populate the EGL. Multiple factors have been shown to contribute to this tangential migration of GNPs over the surface of the cerebellum, including signaling through Slit2/Robo2, Presenilin1 (Psen1), and the Netrin receptor Unc5h3 (Chedotal 2010). GNPs migrating away from the URL express the receptor Robo2 and are repelled by Slits secreted by the rhombic lip, suggesting that Slit/Robo signaling can direct GNP migration away from the rhombic lip (Gilthorpe et al. 2002; Marillat et al. 2002; Geisen et al. 2008). The transmembrane protein Psen1 is thought to promote migration across the surface of the cerebellum, and loss of Psen1 results in a small cerebellum and a failure of GNPs to migrate to the anterior boundary of the cerebellum (Louvi et al. 2004). In contrast, the receptor Unc5h3 is believed to stop migration at this anterior boundary, and transgenic mouse studies have demonstrated that loss of Unc5h3 causes extended migration beyond this region (Przyborski et al. 1998; Goldowitz et al. 2000). The appropriate balance of signaling through these factors is necessary to distribute GNPs across the cerebellar surface, which ultimately enables the proper patterning and population of granule neurons within the mature cerebellum.

1.1.2 Proliferation of GNPs

In conjunction with migration across the surface of the cerebellum, GNPs within the EGL must proliferate extensively to generate the abundant numbers of granule neurons that will ultimately be required for the functioning of the mature cerebellum. While the signals that regulate proliferation versus differentiation of GNPs are not fully
understood, one known mitogen for GNPs is the secreted factor Sonic hedgehog (Shh) (Wechsler-Reya and Scott 1999) (Wallace 1999). The Shh pathway is activated by binding of the Shh ligand, which is secreted by Purkinje cells, to the transmembrane protein Patched1 (Ptch1) on GNPs. In the absence of ligand, Ptch1 functions as a repressor of signaling by blocking the localization of the 7-pass transmembrane protein Smoothened (Smo) to the primary cilium, a cellular structure in which the majority of the downstream Shh pathway components are concentrated (Corbit et al. 2005) (Rohatgi et al. 2007). Upon binding of Shh, this repression is relieved, and Smo translocates to the cilium, where it can then activate the downstream components of the pathway, leading to release of Gli family transcription factors from a complex containing Suppressor of Fused (SuFu). The Gli proteins are then able to translocate to the nucleus where they can activate transcription of target genes, including important regulators of the cell cycle (such as Cyclin D1 and N-myc), therefore promoting cell cycle progression and proliferation.

In addition to Shh, other factors are thought to contribute to GNP proliferation, including signaling through Insulin-like Growth Factor Receptor 1 (Igfr1), Notch2, and Transforming Growth Factor-β2 (Tgf-β2). Igfr1 expression can be detected in the EGL, and treatment of GNPs with the ligands Igf1 or Igf2 has been shown to enhance proliferation and synergize with Shh (Liu et al. 1993; Fernandez et al. 2010). Similarly, the Notch2 receptor is also expressed by GNPs, and exposure to the ligand Jagged1
(Jag1) causes increased GNP proliferation (Solecki et al. 2001). Treatment of GNPs with the secreted molecule Tgf-β2 has also been shown to promote proliferation (Elvers et al. 2005). The appropriate regulation of these pathways is critical to prevent aberrant proliferation that could lead to tumor formation while allowing the generation of the cell numbers required for mature cerebellar function.

1.1.3 Differentiation and radial migration of GNPs

While GNP proliferation in mice continues for the first 2 weeks after birth, subsets of these cells begin to initiate differentiation shortly before birth. However, the signals that regulate the switch from proliferation to differentiation remain largely unknown. Candidate factors include members of the bone morphogenic protein (BMP) family, including BMP2 and BMP4, the secreted molecule basic fibroblast growth factor (bFGF), and the extracellular matrix protein vitronectin. BMP2 and 4 have been shown to be expressed by post-mitotic GNPs, and treatment with these proteins can inhibit Shh-induced GNP proliferation via signaling through Smad1 (Rios et al. 2004). Similarly, treatment of GNPs with bFGF has been shown to inhibit Shh pathway activity and promote differentiation (Fogarty et al. 2007). Vitronectin has also been shown to promote GNP differentiation by downregulating the proliferative response to Shh through phosphorylation of CREB (Pons et al. 2001). Differentiation of GNPs is marked by downregulation of expression of Math1 and other proliferation-associated
transcription factors (including Zic1, Barhl1, and Zipro1) and increased expression of mature granule neuron markers (such as NeuN, Tag1, Tuj1, and NeuroD1).

The initiation of differentiation in GNPs is also marked by a switch from tangential migration across the surface of the cerebellum to radial migration towards the interior of the cerebellum. Differentiating granule neurons extend long, parallel fiber axons and migrate along the Bergmann glial processes that span from the interior of the cerebellum to the surface of the EGL. Cells migrate along these glial processes through the molecular layer (which consists of Purkinje, basket, and stellate neurons) and establish the internal granule layer (IGL) of mature granule neurons.

While the precise signals regulating the switch from tangential to radial migration have yet to be fully understood, multiple factors have been shown to contribute to radial migration. For example, Astrotactin, a molecule secreted by Bergmann glia that contains epidermal growth factor (EGF) repeats and fibronectin repeats, has been shown to act as a heterophilic ligand for glial binding, and Astrotactin-deficient mice display a disruption in granule neuron migration (Hatten 1999) (Zheng et al. 1996) (Adams et al. 2002). Multiple members of the Semaphorin/Plexin family, including Sema6A/Plexin-A2 and Sema4C/Plexin-B2, have also been shown to regulate radial migration. The transmembrane protein Sema6A and its receptor Plexin-A2 are expressed by tangentially migrating GNPs and are downregulated upon the initiation of radial migration (Kerjan et al. 2005). GNPs in Sema6A−/− or Plexin-A2−/− mice have been
shown to fail to initiate radial migration, due to defects in the nucleus-centrosome coupling that is required for migration, and many granule neurons in these animals are unable to reach the IGL (Renaud et al. 2008) (Kerjan et al. 2005). In contrast to the inability of GNPs to migrate radially in Sema6A or Plexin-A2 knockout mice, animals deficient in Sema4C/Plexin-B2 signaling display premature differentiation and radial migration (Friedel et al. 2007) (Deng et al. 2007) (Maier et al. 2011). However, some of these prematurely migrating cells fail to reach the IGL, suggesting that Sema4C/Plexin-B2 can regulate both the timing and trajectory of radial migration.

In addition to the factors described above, a balance between opposing chemoattractants from the interior vs. the surface of the cerebellum is also thought to contribute to the regulation of radial migration. The neurotrophin brain-derived neurotrophic factor (BDNF) is secreted by mature granule neurons in the IGL, and a gradient of BDNF from the IGL is thought to promote the migration of differentiating GNPs out of the EGL towards the interior of the cerebellum (Borghesani et al. 2002) (Yacubova and Komuro 2003). In contrast, the chemokine stromal-derived factor 1 (SDF-1, also known as CXCL12) is secreted by the meninges covering the surface of the cerebellum, and during embryonic stages, the interaction between SDF-1 and its receptor CXCR4 (expressed by GNPs) is thought to maintain cells within the EGL, allowing continued proliferation and preventing premature exit from this mitogenic niche (Zhu et al. 2002) (Vilz et al. 2005) (McGrath et al. 1999). While the mechanisms
that determine response to these opposing guidance forces have yet to be fully understood, the expression of ephrin-B2 and the receptor EphB2 have been shown to increase around the time of the initiation of radial migration, and reverse signaling induced by soluble EphB2-Fc can inhibit the chemoattractant effect of SDF-1 on GNPs, suggesting that EphB2 may act as a switch to mediate a change in responsiveness to SDF-1 vs. BDNF (Lu et al. 2001). Although the SDF-1/CXCR4 axis has been shown to be necessary for proper GNP migration during embryonic stages, its role in postnatal GNP migration remains unknown. Further examination of the influence of this pathway will be important for better understanding its contribution to cerebellar development.

1.1.4 SDF-1/CXCR4 signaling

The SDF-1 ligand has been shown to be expressed in many tissues, including lung, liver, skeletal muscle, brain, heart, and bone marrow, and is secreted by endothelial precursor cells (McGrath et al. 1999) (Sun et al. 2010). During development, SDF-1 primarily contributes to hematopoiesis and heart and brain development and vascularization (Ma et al. 1998) (Nagasawa et al. 1996). SDF-1 functions by binding to the seven-span transmembrane G protein-coupled receptor CXCR4 and activating signaling through intracellular heterotrimeric G-proteins. Activation of CXCR4 has been shown to result in inhibition of adenylyl cyclase-mediated cyclic adenosine monophosphate (cAMP) production and an increase in intracellular calcium, as well as activation of the phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase
(MAPK), and JAK/Stat signaling pathways (Teicher and Fricker 2010). Like SDF-1, CXCR4 is also widely expressed in many tissues and cell types throughout the body, and loss of CXCR4 during development has been shown to result in defects in hematopoietic, heart, and brain development (Wegner et al. 1998) (Zou et al. 1998) (Miller et al. 2008) (Tissir et al. 2004).

The SDF-1/CXCR4 system has been shown to mediate migration and invasion in many contexts. Gradients of SDF-1 can attract circulating CXCR4-expressing cells to target locations during both normal development and homeostasis as well as during tumor progression. For example, SDF-1 is thought to contribute to hematopoiesis by promoting homing of CXCR4-expressing hematopoietic stem cells to the bone marrow, and patients requiring autologous bone marrow transplants have been treated with the CXCR4 antagonist AMD3100 to mobilize hematopoietic stem cells from the bone marrow into the blood, allowing the isolation of the stem cells for transplantation (Suarez-Alvarez et al. 2012). SDF-1 has also be shown to promote neoangiogenesis by mobilizing and recruiting CXCR4-expressing proangiogenic cells (such as hematopoietic cells, endothelial precursor cells, and smooth muscle progenitor cells) to sites of new blood vessel formation, such as in the revascularization of ischemic tissue or in tumor growth (Petit et al. 2007). Similarly, the chemoattractant effects of SDF-1 have been shown to promote the metastasis of CXCR4-overexpressing tumor cells in many types of cancer to distant sites of SDF-1 expression, such as lymph nodes, lung, liver, or bone
(Darash-Yahana et al. 2004) (Lievens et al. 2010). Hypoxia has been shown to cause an upregulation in the expression of CXCR4, which may enhance the ability of tumor cells to migrate to these sites (Schioppa et al. 2003). CXCR4 has also been shown to promote the proliferation and survival of tumor cells through activation of the PI3K and MAPK pathways, and CXCR4 antagonists are currently in clinical trials for cancer treatment (Peled et al. 2012) (Khan et al. 2007).

1.1.4.1 SDF-1/CXCR4 in the central nervous system

The SDF-1/CXCR4 axis has been implicated in the development of the central nervous system. SDF-1, expressed by the meninges or mesenchymal cell types, has been shown to act as a chemoattractant for migratory neurons in the dentate gyrus, cerebral cortex, dorsal root ganglia, brainstem nuclei, and cerebellum (Stumm and Hollt 2007). In the dentate gyrus, progenitor cells migrate along a migratory stream from the wall of the lateral ventricle to form a germinal matrix. A gradient of expression of SDF-1 from the meninges is thought to promote this movement of CXCR4-expressing progenitor cells along the migratory stream, and loss of the CXCR4 causes cells to stall in this stream (Bagri et al. 2002; Lu et al. 2002; Tran and Miller 2003). Similarly, migration of interneuron progenitors from the ganglionic eminence to the cerebral cortex and radial migration of Cajal–Retzius cells from the cortical ventricular zone toward the cortical surface have also been shown to be defective in CXCR4-deficient mice (Stumm and Hollt 2007). The SDF-1/CXCR4 axis has also been reported to contribute to repair after brain
injury, such as stroke. SDF-1 expressed at the site of injury can recruit the migration of CXCR4-expressing neuronal progenitor cells to the site of injury, allowing repair of the damaged tissue (Robin et al. 2006; Thored et al. 2006).

In the cerebellum, SDF-1/CXCR4 has been shown to regulate the migration of GNPs during embryonic development (Ma et al. 1998; Zou et al. 1998; Klein et al. 2001). SDF-1 and CXCR4 knockout mice show identical phenotypes, with premature radial migration of a subset of proliferating GNPs from the EGL to the interior of the cerebellum. However, these mice do not survive past birth, likely due to defects in cardiac septum formation, which has prevented analysis of the fate of these prematurely migrating cells and the role of SDF-1/CXCR4 signaling in postnatal cerebellar development. Because a significant period of cerebellar development continues after birth, assessing the contributions of SDF-1/CXCR4 signaling during this period will be important for fully understanding the mechanisms that contribute to the formation of the mature cerebellum.

1.1.4.2 Role of SDF-1/CXCR4 in postnatal cerebellar development

Much of what is known about cerebellar development has come from analysis of loss of function mouse models. However, the majority of these models have utilized conventional gene knockout approaches, such that the observed effects are often consequences of embryonic loss of function in all of the cell types in the cerebellum. Few studies of genes involved in GNP proliferation, differentiation, and migration have
specifically examined the effects of disrupting signaling specifically in GNPs. In Chapter 2, I will describe our efforts to understand the consequences of disruption of SDF-1/CXCR4 signaling on postnatal cerebellar development, using conditional Cre-mediated deletion of CXCR4 specifically in GNPs. We show that similar to the embryonic phenotype, loss of CXCR4 leads to premature migration of a subset of postnatal GNPs that continue to proliferate outside of the EGL. These cells also migrate beyond their normal destination in the IGL, but survive and differentiate appropriately into mature granule neurons regardless of their location. Our data suggest that CXCR4 is required for appropriate radial migration, but not proliferation or differentiation, throughout cerebellar development.

1.2 Tumorigenesis

Aberrant regulation of the mechanisms governing cerebellar development can lead to tumor formation. Medulloblastoma (MB), a class of tumors that arise in the cerebellum, is the most common malignant brain tumor in children. The disease is usually treated by surgical resection followed by radiation and chemotherapy. While this regimen has been relatively effective at eradicating the tumor (with 5-year survival rates of up to 80%), survivors often suffer severe physical, cognitive, and emotional deficits (Crawford et al. 2007) (Rossi et al. 2008) (Saury and Emanuelson 2011) (Wolfe-Christensen et al. 2007) (Xu et al. 2004). Improved therapeutic approaches are clearly
necessary and are likely to come from a deeper understanding of the molecular basis for this disease.

1.2.1 Medulloblastoma subtypes

Tumors classified as MB can differ markedly from one another in terms of histology, genetics, prognosis and therapeutic responsiveness. Historically, MB has been histologically classified into four major subtypes: desmoplastic, classic, large cell/anaplastic (LC/A), and MB with extensive nodularity (MBEN) (Gulino et al. 2008) (Lamont et al. 2004). The desmoplastic subtype represents approximately 25% of MBs and is characterized by nodules of sparsely-distributed differentiated cells, often bounded by extracellular matrix (reticulin), and interspersed with regions of more densely-packed proliferating cells (McManamy et al. 2007). The “classic” subtype, which is comprised of relatively uniform sheets of undifferentiated small round blue cells, represents approximately 65% of MB cases. LC/A tumors consist of large, heterogeneous cells with prominent nucleoli, nuclear wrapping and high mitotic and apoptotic indices, represent approximately 5% of MB cases, and are associated with extremely poor prognosis (von Hoff et al. 2010) (Eberhart and Burger 2003) (Leonard et al. 2001). Finally, MBEN tumors, which display greater nodularity and more advanced neuronal differentiation than desmoplastic tumors, represent 5% of all MBs and have a relatively favorable prognosis (Giangaspero et al. 1999).
Although patient stratification of MB has historically been based upon histological classification, recent genomic data have significantly enhanced the ability to classify tumors, define key molecular alterations, and begin to develop appropriate targeted therapies (Taylor et al. 2012) (Northcott et al. 2010) (Cho et al. 2010b) (Kool et al. 2008) (Thompson et al. 2006). Expression profiling (mRNA and miRNA), DNA copy number analysis, and mutational analysis have enabled classification of MB into at least four molecular subtypes, including SHH-associated, WNT-associated, “Group 3” (characterized by overexpression or amplification of the MYC oncogene) and “Group 4” (a heterogeneous group of tumors for which a defining molecular event has not yet been identified). In addition to their differences in genetic alterations, these subtypes also differ in clinical features, such as age and gender distribution, likelihood of metastasis and patient outcome.

1.2.1.1 SHH-associated subtype of medulloblastoma

Approximately 20-30% of MBs are associated with mutation or activation of the SHH pathway (Northcott et al. 2010) (Cho et al. 2010b) (Kool et al. 2008) (Thompson et al. 2006). The importance of this pathway in MB was first recognized when Gorlin syndrome, a hereditary disease associated with increased incidence of basal cell carcinoma and MB, was linked to germline mutations in PTCH1 (Evans et al. 1991) (Johnson et al. 1996) (Hahn et al. 1996). Inactivating mutations in PTCH1 and SUFU, activating mutations in SMO, and amplification of GLI2 have also been observed in
sporadic MBs (Cho et al. 2010b) (Pietsch et al. 1997) (Raffel et al. 1997) (Taylor et al. 2002) (Lam et al. 1999). SHH-associated tumors occur predominantly in infants and adults, but not in children, and are associated with a mixed prognosis (Northcott et al. 2010) (Cho et al. 2010b). These tumors are thought to arise from aberrant developmental regulation of GNPs within the EGL, and many tumors continue to display GNP-like characteristics, including expression of the transcription factor ATOH1 (MATH1). However, the alterations that cooperate with SHH pathway activation in MB have yet to be fully elucidated. The recent improved understanding of the molecular properties of this subtype represents a significant advance that will enable more accurate diagnosis and implementation of appropriate treatments for patients.

1.2.1.2 Mouse models for SHH-associated subtype

The recent genomic data have generated numerous hypotheses regarding the biology and appropriate treatments for each of the subtypes of MB. One approach to testing these hypotheses is using genetically engineered mouse models, which can provide valuable information about the biology of the disease as well as platforms for the evaluation of potential therapies.

One of the most widely utilized mouse models of MB is the Ptch1-knockout mouse (Goodrich et al. 1997). These animals were created using conventional homologous recombination technology, which replaced a portion of the Ptch1 gene with LacZ and neomycin reporter genes, leading to loss of expression of the Ptch1 protein.
Because Ptch1 functions as a repressor of SHH signaling, the pathway is constitutively active in these mice. Homozygous (Ptch1⁻/⁻) mice exhibit severe defects in the neural tube, heart and other tissues, and die during embryogenesis. In contrast, heterozygotes (Ptch1⁺/⁻ mice) are viable, and 15-20% develop cerebellar tumors that resemble human MB (Goodrich et al. 1997) (Hahn et al. 2000). These mice have been used extensively to examine properties of MB driven by SHH pathway activation, such as the early stages of tumorigenesis, cooperating oncogenic pathways, the role of cancer stem cells, the mechanisms of radioresistance, and the effects of targeted therapies (Oliver et al. 2005) (Kessler et al. 2009) (Wetmore et al. 2001) (Uziel et al. 2005) (Briggs et al. 2008) (Zhao et al. 2008) (Read et al. 2009) (Ward et al. 2009) (Hambardzumyan et al. 2008) (Romer and Curran 2005) (Buonamici et al. 2010).

Conditional Ptch1 knockout models of MB have also recently been developed (Yang et al. 2008b). In these animals, loxP-flanked alleles of Ptch1 (Ptch1floxed) are deleted by Cre recombinase, whose expression is controlled by the Math1 or glial fibrillary acidic protein (GFAP) promoters. The resulting mice develop MB with an incidence of 100% and a much shorter average latency than conventional Ptch1⁺/⁻ mice. Given this high tumor incidence and short latency, this model has proven useful for preclinical studies.

Activation of other components of the SHH pathway (such as constitutive expression of exogenous SHH ligand or activating mutations in SMO) has also been
shown to cause MB in mice, and additional mouse models have identified cooperating oncogenes or tumor suppressors (including c-MYC, N-myc, insulin-like growth factor 2 (Igf-2), Akt, Bcl-2, or hepatocyte growth factor (HGF), or loss of p53, Ink4c, Kip1, p19-Ink4d) (Binning et al. 2008) (Browd et al. 2006) (Broderick et al. 2004) (Rao et al. 2003) (Rao et al. 2004) (Hatton et al. 2008) (Hallahan et al. 2004) (Lee et al. 2007) (Heby-Henricson et al. 2011) (McCall et al. 2007) (Coon et al. 2010). These models have proven valuable for understanding the biology of MB and assessing potential therapies for the treatment of SHH-associated MB. For example, hedgehog antagonists have recently been developed for the treatment of SHH-associated tumors, and mouse models have been instrumental in testing the efficacy of these compounds (Tremblay et al. 2009) (Gould and Missailidis 2011) (Low and de Sauvage 2010) (Berman et al. 2002) (Romer et al. 2004) (Buonamici et al. 2010). As a result of promising outcomes in preclinical studies, patients are now being treated with SHH antagonists. Unfortunately, although response to these agents has been observed, patients can quickly develop resistance to this targeted therapy (Low and de Sauvage 2010) (Rudin et al. 2009) (Yauch et al. 2009) (Metcalfe and de Sauvage 2011). Mouse models are now being used to understand the mechanisms of this resistance and to identify approaches to preventing or overcoming it (Dijkgraaf et al. 2011) (Buonamici et al. 2010).
1.2.2 Tumor-propagating cells

One approach to identifying improved strategies to treat tumors and overcome therapeutic resistance is through targeting tumor-propagating cells (TPCs). Tumor-propagating cells, also called “cancer stem cells” have been defined as subsets of cells within a tumor that have a unique capacity to maintain and propagate tumor growth. TPCs were first identified in the context of acute myeloid leukemia, where it was found that specific populations of leukemia cells (prospectively isolated based on expression of cell surface markers, such as CD34+/CD38-) were capable of re-establishing the full leukemia upon transplantation of the population into naïve immunocompromised mice (Bonnet and Dick 1997). This observation suggested the existence of heterogeneity within the leukemia, such that not all tumor cells were equivalent in terms of their tumorigenic capacity. In the context of leukemia, the TPCs were found to display properties of normal hematopoietic stem cells and the potential to differentiate into other committed cell types, thus leading to the term “cancer stem cell”. However, the cancer stem cell hypothesis does not require TPCs to display stem cell properties and does not imply a stem cell origin of the tumor, but instead refers to the unique capacity of populations of cells within a tumor to regenerate the tumor.

After the identification of TPCs within hematopoietic malignancies, investigators began to assess whether solid tumors also contain unique populations of TPCs. Initial observations from brain and breast cancer confirmed that solid tumors can indeed
contain these cells, and tumor-propagating populations have now also been identified in tumors of the colon, pancreas, liver, lung, prostate, and skin, among others (Al-Hajj et al. 2003) (Singh et al. 2004) (O’Brien et al. 2007) (Li et al. 2007) (Xin et al. 2005) (Bertolini et al. 2009) (Yang et al. 2008a) (Schober and Fuchs 2011). However, the cell surface markers that enable prospective isolation of the TPCs can differ between tumors. While CD133 (Prominin1) has been shown to be a common marker for tumor-propagating populations in many tumor types (including brain, lung, colon, and pancreatic tumors, among others), other cell surface antigens, such as CD44, CD24, CD90, Lgr5, CXCR4, CD29, and CD15, have also been shown to mark cells with tumor-propagating potential in various tumor types others (Al-Hajj et al. 2003) (Singh et al. 2004) (O’Brien et al. 2007) (Li et al. 2007) (Xin et al. 2005) (Bertolini et al. 2009) (Yang et al. 2008a) (Schober and Fuchs 2011). In many cases, these cells have been shown to exhibit resistance to radiation and chemotherapeutics, leading to the notion that TPCs are responsible for the most malignant properties of the tumors (McCubrey et al. 2012). Therefore, specifically targeting this population may represent a less toxic therapeutic approach for killing those cells with the capacity to regenerate tumors after therapy.

1.2.2.1 Tumor-propagating cells in brain tumors

Human brain tumors were among the first solid tumors shown to contain tumor-propagating cell populations (Singh et al. 2004) (Singh et al. 2003) (Hemmati et al. 2003). In the initial studies, primary patient tissues from human glioblastoma and
medulloblastoma were dissociated, sorted into populations based on expression of CD133, then injected into the brains of immunocompromised mice. Although cells from both the CD133+ and CD133- populations demonstrated long-term engraftment and survival in the host mice, only the CD133+ population was capable of regenerating tumors that resembled the primary tumors from which they were derived. Further studies have confirmed that CD133 can often mark the tumor-propagating population in many brain tumors, but in some cases, this capacity resides in the CD133- population, suggesting that heterogeneity exists between tumors, and the TPC population may differ between tumors. Recent studies have suggested that integrin α6, CD15 (SSEA-1), and the ganglioside A2B5 may also enrich for TPCs in human glioblastoma (Son et al. 2009) (Lathia et al. 2010) (Ogden et al. 2008) (Tchoghandjian et al. 2010).

Regardless of the marker used for identification, the ability of TPCs to regenerate tumors has led to the notion that these cells are responsible for tumor recurrence after therapy in patients. Brain tumor propagating cells have been shown to display resistance to both radiation and chemotherapy (Bao et al. 2006) (Liu et al. 2006). Irradiation of glioma cells has been shown to cause an accumulation of CD133+ TPCs, and these cells display an enhanced capacity for DNA damage repair, leading to their ability to evade the toxic consequences of irradiation. Similarly, CD133+ cells have also been shown to display decreased susceptibility to the DNA alkylating agent temozolomide, a standard chemotherapeutic used in clinical treatment of glioma. In
addition to differences in DNA damage repair response, the differential sensitivities of TPCs to chemotherapy (compared to the majority of cells within the tumor bulk) have also been shown to result from differences in efflux of the drugs from the cells, the balance between pro- vs. anti-apoptotic proteins, the expression of metabolic mediators, and the microenvironmental niche of the tumor-propagating cells (Frame and Maitland 2011) (Donnenberg and Donnenberg 2005). Therefore, given this capacity for evading standard therapies and regenerating the tumor, the identification of novel therapeutic approaches to target and eliminate these cells is critical for improving patient treatment outcomes.

1.2.2.2 Tumor-propagating cells in SHH-associated medulloblastoma

Studies of human medulloblastoma have indicated that tumor-propagating potential could reside in either the CD133+ or CD133- populations, depending on the individual tumor. However, the limited availability of primary patient tissues has hindered full understanding of these cells or identification of approaches to target them. To overcome this challenge, our lab has employed mouse models for the study of medulloblastoma TPCs. Using patched heterozygous (Ptch+/−) mice, we have previously identified the cell surface antigen CD15 as a tumor-propagating cell marker in tumors arising in these mice (Read et al. 2009). CD15, also known as Lewis X antigen or SSEA-1, is a trisaccharide carbohydrate antigen that is expressed on a variety of cell types, including neutrophils, brain stem or progenitor cells, and murine pluripotent stem cells.
(Capela and Temple 2002; Yanagisawa et al. 2005) (Stocks et al. 1990) (Warren et al. 1996) (Muramatsu 1988) (Muramatsu and Muramatsu 2004). While the CD15 carbohydrate is generally believed to be involved in adhesion and chemotaxis, its function in Ptch\(^{+/−}\) tumors remains unknown (Hakomori 1992; Boubelik et al. 1998; Handa et al. 2007; Lieberoth et al. 2009). However, expression of CD15 defines the cellular population that is capable of propagating Ptch\(^{+/−}\) tumors; when CD15+ cells are transplanted into the cerebella of naïve mice, 100% of the animals develop tumors, whereas CD15- cells never give rise to tumors. CD15+ cells from Ptch\(^{+/−}\) tumors have been shown to be more proliferative than CD15- cells, and microarray expression analysis has indicated decreased expression of genes associated with differentiation in the CD15+ population and elevated expression of genes associated with proliferation. Furthermore, a subset of human medulloblastomas express CD15, and a CD15-associated gene signature (based on expression of genes that differentiate CD15+ from CD15- population in Ptch\(^{+/−}\) tumors) has been shown to be associated with poorer prognosis in these tumors (Read et al. 2009).

1.2.3 Targeting CD15+ tumor-propagating cells

Our identification of the CD15+ tumor-propagating population in Ptch\(^{+/−}\) tumors has enabled us to initiate efforts to identify approaches for targeting these cells. One such approach involves gaining insight into the role of the CD15 epitope or associated carrier molecule and developing targeting strategies based on expression of the epitope.
In Chapter 3, I will discuss our efforts to identify the carrier molecule for the CD15 carbohydrate using a biochemical immunoprecipitation approach as well as an antibody-drug conjugate immunotoxin approach for targeting the CD15+ population. Unfortunately, we were unable to identify specific CD15 carrier molecules, but our data suggest that the carbohydrate is expressed on both proteins and lipids in Ptch+/− tumor cells, and future studies should examine the contribution of both types of CD15-associated molecules to tumorigenesis. We also show that the immunotoxin-based targeting approach could not be used to specifically target CD15+ cells, due to non-specific toxicity of the control immunotoxin in Ptch+/− tumor cells. Future immunotoxin-based approaches for targeting these cells may require the use of alternative antibody isotypes.

Chapter 4 will describe an alternative approach for targeting CD15+ TPCs based on the identification of differential cell cycle kinetics and expression of cell cycle regulators in CD15+ and CD15- cells. We show that CD15+ cells progress more rapidly through the cell cycle and express elevated levels of the Aurora and Polo-like kinases. Treatment of Ptch+/− tumor cells or patient-derived medulloblastoma xenograft cells with small molecule inhibitors of these kinases could block tumor growth both in vitro and in vivo, suggesting that targeting this property of CD15+ cells may represent an approach for improving treatment of SHH-associated medulloblastoma. These studies have shed light on the properties of CD15+ tumor-propagating cells in medulloblastoma and have
identified new approaches for targeting these cells and improving treatment for patients with this devastating disease.
2. CXCR4 is required for appropriate radial migration of cerebellar granule neuron progenitors

2.1 Introduction

The formation of the mature nervous system requires exquisite organization. Neurogenesis involves both the expansion of neuronal precursors and the migration of mature neurons to their ultimate location in the developing brain. Although these processes are absolutely essential for the establishment of properly functioning neurons, further investigation is required to understand the signals that control neuronal proliferation and migration during development.

The developing cerebellum represents an ideal context in which to study the factors that regulate neuronal proliferation and migration. Development of granule neurons, the most abundant cell type in the cerebellum, is known to proceed through a relatively well-established sequence of proliferation, migration, and differentiation (Hatten and Roussel 2011). Cells originating from the neuroepithelium of the ventricular zone migrate over the rhombic lip to become granule neural progenitors (GNPs) (Wang and Zoghbi 2001; ten Donkelaar et al. 2003). These cells then migrate tangentially over the surface of the cerebellum, forming a layer of GNPs known as the external granule layer (EGL). The EGL expands by GNP proliferation throughout embryonic stages, then ultimately shrinks and disappears as GNPs migrate radially to form the internal granule layer (IGL) of mature granule neurons. While the signals that control GNP migration and development are not well understood, this proliferative
region on the surface of the cerebellum is considered to be a mitogenic niche for GNPs (Choi et al. 2005). Within this region, contact with the meninges is thought to contribute to maintaining GNPs in proximity to the secreted mitogenic factor Shh, which allows the proper proliferation and expansion of GNPs required to ultimately generate the appropriate number of granule neurons within the cerebellum (Zhu et al. 2002; Zhu et al. 2004). While multiple factors, including BDNF, Astrotactin, Semaphorins/Plexins and Ephrins, are thought to contribute to the regulation of appropriate migration away from the EGL upon GNP differentiation, the mechanisms controlling this process remain incompletely understood (Lu et al. 2001; Adams et al. 2002; Borghesani et al. 2002; Renaud et al. 2008; Maier et al. 2011).

One important factor that contributes to regulating migration is the secreted factor SDF-1. SDF-1 (also known as CXCL12), a chemokine produced by meningeal cells, is the ligand for the G protein-coupled receptor CXCR4 (McGrath et al. 1999; Vilz et al. 2005). Because GNPs, but not mature granule cells, express high levels of CXCR4, SDF-1 from the meninges is thought to act as a chemoattractant contributing to both the establishment of the EGL on the surface of the cerebellum and maintenance of this region until GNPs are ready to migrate and differentiate (Klein et al. 2001). In addition, SDF-1 has been shown to synergize with the mitogen Sonic hedgehog (Shh) to promote proliferation of these cells, thus functioning as both a chemoattractant and a mitogenic factor important for the expansion of progenitor cells in the EGL (Klein et al. 2001).
Previous studies using CXCR4+/− and SDF-1+/− mice have confirmed the chemoattractant activity of SDF1 during embryonic cerebellar development (Ma et al. 1998; Zou et al. 1998). While GNP inward migration and differentiation normally begins around birth, these mice display premature migration defects in which a subset of GNPs migrates to the interior of the cerebellum during embryonic stages. These aberrantly migrating cells are unusual in that they express the GNP marker Math1 and proliferate outside of the EGL mitogenic niche. These data suggest that SDF-1/CXCR4 signaling contributes to regulating the appropriate timing of embryonic GNP migration, but because these mice die perinatally, the fate of these prematurely migrating cells and the consequences of loss of CXCR4 signaling on postnatal cerebellar development have remained unknown.

CXCR4 signaling is also thought to contribute to a number of malignancies, including the cerebellar tumor medulloblastoma (Darash-Yahana et al. 2004; Schuller et al. 2005). In many solid tumors, signaling between SDF-1 and CXCR4 is believed to promote metastasis, as tumor cells expressing CXCR4 migrate towards blood vessels expressing SDF-1 (Lievens et al. 2010). This CXCR4-mediated chemoattraction thus enables eventual extravasation and metastasis of the tumor cells. However, the role of CXCR4 in medulloblastoma is unclear. Tumors in the SHH-associated subset of human medulloblastoma have been shown to express high levels of CXCR4, and treatment of medulloblastoma cell lines and xenografts with the CXCR4 antagonist AMD3100 has
been shown to inhibit growth (Rubin et al. 2003; Schuller et al. 2005; Yang et al. 2007; Sengupta et al. 2012). However, the influence of CXCR4 signaling on tumor initiation has remained unclear.

To investigate the role of CXCR4 in postnatal cerebellar development and tumorigenesis, we generated conditional knockout mice in which CXCR4 expression is eliminated specifically in GNPs. These mice survive to adulthood and show no gross phenotypic abnormalities. We show that these mice display premature migration defects and reduced Shh pathway activity in GNPs, but are capable of appropriate proliferation and differentiation. Furthermore, loss of CXCR4 does not affect tumor formation in patched mutant mice. Our data suggest that while SDF-1/CXCR4 signaling is dispensable for granule cell proliferation, differentiation, and tumorigenesis, it is critical for proper migration of cerebellar GNPs.

### 2.2 Methods

#### 2.2.1 Mice

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committees of Duke University and the Sanford-Burnham Medical Research Institute. Math1-Cre and Math1-CreER mice were obtained from the Jackson Laboratory (Bar Harbor, ME). CXCR4\textsuperscript{flox/flox} mice were a kind gift from D. Littman (Nie et al. 2004). Math1-Cre; CXCR4\textsuperscript{flox/flox} or CXCR4\textsuperscript{flox/flox} mice were collected
at embryonic day 17.5 or at post-natal days 8, 14, 21 or 8 weeks to examine the effects of CXCR4 loss on cerebellar development. Conditional Math1-CreER; CXCR4\textsubscript{flox/flox} mice were treated with 0.6 mg of tamoxifen (T5648, Sigma, St. Louis, MI) in 30 \(\mu\)l of corn oil at post-natal days 0, 1, 2, 4, or 5, and tissue was harvested at postnatal days 7, 10 or 14 to examine the consequences of CXCR4 deletion. Math1-Cre; Ptc\textsubscript{floxflox} have previously been described by our laboratory (Yang et al. 2008b), and Math1-Cre; Ptc\textsubscript{floxflox}; CXCR4\textsubscript{flox/flox} mice were generated to examine the effects of CXCR4 deletion on tumorigenesis.

2.2.2 Tissue collection

Cerebella were harvested at multiple embryonic and postnatal timepoints to assess the effects of CXCR4 deletion on cerebellar development. For embryonic timepoints, embryos were harvested and placed in 4% paraformaldehyde (PFA; Sigma) for fixation overnight at 4°C. For postnatal timepoints, the animals received a lethal dose of anesthesia and were perfused with cold phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA) followed by 4% PFA through the left ventricle. Cerebella were obtained by dissection and further fixed overnight in 4% PFA. After fixation, all samples were transferred to a solution of 30% sucrose (Sigma) for 2 days, then embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA). The samples were then stored at -80°C until sectioning (10 \(\mu\)m) on a Leica CM3050S Cryostat (Leica Microsystems, Inc., Buffalo Grove, IL).
2.2.3 Immunostaining

To determine the effects of CXCR4 deletion on cerebellar development, frozen tissue sections were immunostained for markers of proliferation and differentiation. Sections were blocked and permeabilized for 1 hour with PBS containing 0.1% Triton X-100 and 1% normal goat serum, stained with primary antibodies overnight at 4°C, and incubated with secondary antibodies for 45 minutes at room temperature. Primary antibodies used for immunostaining included NeuN (1:100) and rabbit anti-GABRA6 (1:100) from Chemicon/Millipore (Temecula, CA); mouse anti-Ki67 (1:100), rabbit anti-BLB (1:500), and mouse-anti-BrdU (clone 3D4) from BD Biosciences (San Jose, CA), and mouse anti-Calbindin-D28K (1:250) from Sigma. Secondary antibodies included Alexa Fluor-568 anti-mouse IgM (1:200), Alexa Fluor-594 anti-rabbit IgG (1:200) and Alexa Fluor-488 anti-mouse IgG (1:200) from Invitrogen. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) and mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL). Images were acquired using a Zeiss Axio Observer microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) and MetaMorph 7.6 software (Molecular Devices, Sunnyvale, CA).

2.2.4 In situ hybridization

Animals were perfused with 4% PFA and brains were embedded in OCT and cut into 12 μM sections. Sections were fixed in 4% PFA, acetylated and incubated for 1 hour at room temperature in pre-hybridization buffer (50% formamide, 5X SSC, 1X...
Denhardt’s, 250 μg/ml yeast tRNA, and 500 μg/ml 1 herring sperm DNA; all from Sigma). Sections were hybridized overnight at 65°C in hybridization buffer (50% deionized formamide, 1X Denhardt’s, 300 mM NaCl, 20 mM Tris- HCl, pH 8.0, 5 mM EDTA, 10 mM Na₂HPO₄, pH 7.4, 10% dextran sulfate, and 0.5 mg/ml yeast tRNA; all from Sigma) containing digoxigenin (DIG)-UTP-labeled probes for Math1. Probes were synthesized using a DIG labeling kit (Roche Molecular Systems, Pleasanton, CA). After hybridization, sections were incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG antibodies (Roche). Bound probe was visualized by incubating slides in nitro-blue tetrazolium/5-bromo-4-chloro-3’-indolyphosphate (NBT/BCIP, Roche) overnight in the dark. Coverslips were mounted with Aqua-Polymount (Polysciences, Warrington, PA).

2.2.5 Isolation of GNPs

Cerebella were collected from Math1-Cre; CXCR4floxflox or CXCR4floxflox mice and minced for dissociation. The complete dissociation procedure has previously been described (Oliver et al. 2005) (Wechsler-Reya and Scott 1999). Briefly, tumors were digested in a papain solution (Worthington Biochemical Corporation, Lakewood, NJ) to obtain a single-cell suspension, then centrifuged through a 35%-65% Percoll gradient (Amersham Biosciences, Piscataway, NJ). GNPs from the 35%-65% interface were suspended in NB-B27(Neurobasal medium with 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin/streptomycin, and B27 supplement, all from Invitrogen) plus 1%
FBS (Invitrogen) for culture. The cells were plated on poly-D-lysine (Sigma)-coated plates.

### 2.2.6 Real-time PCR

Real-time PCR was performed to assess the levels of expression of CXCR4 and Shh target genes in control Math1-Cre; CXCR4<sup>fl/fl</sup> mice. RNA was prepared using an RNeasy kit (QIAGEN, Inc., Valencia, CA). First-strand cDNA was synthesized from equal amounts of RNA (0.1–1 mg) using SuperScript III Reverse Transcriptase (Invitrogen). Triplicate reactions were prepared using a 25 μl mixture containing iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Real-time quantification was performed on a Bio-Rad iCycler iQ system. The following primers were used: *Actin* (F, TATTGGCAACGAGCGGTTCC; R, GGCATAGAGGTCTTACGGATGTC), *CXCR4* (F, GAACCCTGCTTCCGGGATGAA; R, CCAGAAGGGGAGTGTGATGACAA), *Gli1* (F, TTATGGAGCAGCCAGAGACCAG; R, ATGGAGAGAGCCCGCTTCTTTG), *Gli2* (F, TTCATGGAGTCCCAGCAGAA; R, CTGGCCATAGTAGTATAGCG), *N-myc* (F, AACAAGGCCGTAAACCACCTTCAC; R, TGCTGCTGATGGATGGGAAC), and *Cyclin D1* (F, ACACCTTCTCTCCAAAATGC; R, GGGTGGGTTGGAAATGAAC). Analysis was performed using the delta delta Ct method.

### 2.2.7 Proliferation assays

To examine the proliferative capacity of CXCR4-deficient GNPs, cells were isolated as described above and plated in 96-well plates at a density of 0.2 million cells
per well. Cells were immediately pulsed with [methyl-3H]thymidine (Amersham/GE Healthcare, Piscataway, NJ) and cultured in triplicate wells in the presence of 3 μg/ml recombinant Shh protein (PeproTech, Rocky Hill, NJ) for 4 or 12 hours. Cells were harvested onto filters using a Mach IIIIM Manual Harvester 96 (Tomtec, Hamden, CT), and incorporated radioactivity was quantified by liquid scintillation spectrophotometry on a Wallac MicroBeta scintillation counter (PerkinElmer, Waltham, MA).

2.2.8 In vitro response to Shh

To examine whether CXCR4-deficient GNPs can proliferate properly in response to the mitogen Shh, cells from Math1-Cre; CXCR4<sup>flex/flex</sup> or CXCR4<sup>flex/flex</sup> mice were isolated from P6-P8 pups and plated onto poly-D-lysine-coated coverslips in 24-well plates. The cells were cultured as described above in the presence of 3 μg/ml recombinant Shh protein for 48 hours, then fixed with 4% PFA for 20 minutes in preparation for immunostaining. The coverslips were blocked and permeabilized for 1 hour with PBS containing 0.1% Triton X-100 and 1% normal goat serum, stained with an anti-Ki67 primary antibody (BD Biosciences) overnight at 4°C, and incubated with an anti-mouse IgG-Alexa 594 secondary antibody (Invitrogen) for 45 minutes at room temperature. The coverslips were then incubated with DAPI, washed, and mounted onto slides for microscopy. Images were acquired using a Zeiss Axio Observer microscope and MetaMorph 7.6 software.
2.2.9 Tumor formation

To determine whether loss of CXCR4 affects tumorigenesis in Shh-associated tumors, CXCR4\textsuperscript{flox/flox} mice were crossed to Math1-Cre; Ptcflox/flox mice to generate Math1-Cre; Ptcflox/flox; CXCR4\textsuperscript{flox/flox} animals with homozygous deletion of both patched and CXCR4. These animals and control Math1-Cre; Ptcflox/flox animals were monitored for symptoms of tumors and sacrificed upon evidence of illness. Kaplan-Meier survival curves were generated using the software program GraphPad Prism (GraphPad Software, Inc., La Jolla, CA), and statistical significance was assessed using a Gehan-Breslow-Wilcoxon Chi Square test.

2.3 Results

2.3.1 CXCR4 deletion causes premature GNP migration away from the EGL into the IGL and white matter.

The SDF-1/CXCR4 signaling axis has previously been shown to regulate cerebellar GNP migration through analysis of conventional CXCR4 and SDF1 knockout mice, but the perinatal lethality of these mice prevents analysis of the ultimate postnatal consequences of disruption of CXCR4 signaling or the fate of affected cells (Ma et al. 1998; Zou et al. 1998). To determine the postnatal outcome of loss of CXCR4 signaling, we utilized conditional CXCR4\textsuperscript{flox/flox} knockout mice. In these mice, loxP sites flank exon 2 of CXCR4, which represents the majority of the coding sequence for the protein (Nie et
al. 2004). We crossed these mice with Math1-Cre mice, in which expression of Cre recombinase is driven by the Math1 enhancer, which is active specifically in GNPs in the cerebellum but not in mature granule cells or other cerebellar cell types. Because Cre is expressed as early as embryonic day E9.5 in Math1-Cre mice, excision occurs very early in cerebellar development in Math1-Cre; CXCR4\textsubscript{floxed/floxed} mice, and CXCR4 expression is thus lost at all stages of GNP expansion and maturation.

Math1-Cre; CXCR4\textsubscript{floxed/floxed} mice develop normally, survive to adulthood, and show no major defects in the size of the cerebellum or motor coordination (data not shown).

To examine the effects of loss of CXCR4 on cerebellar structure, we collected tissue sections from control CXCR4\textsubscript{floxed/floxed} mice (which do not express Cre recombinase) and Math1-Cre; CXCR4\textsubscript{floxed/floxed} mice at multiple time points and stained the sections with DAPI to label cells and assess any defects resulting from loss of CXCR4 signaling. As shown in Figure 2.1, Math1-Cre; CXCR4\textsubscript{floxed/floxed} mice display ectopic cells associated with the EGL at embryonic stages, which is consistent with the conventional CXCR4\textsuperscript{−/−} embryonic phenotype (Zou et al. 1998). Similar groups of ectopic cells remain at early postnatal stages (P8 and P14), and clusters of these cells can be seen associated with the EGL at the surface of the cerebellum and extending inward toward the IGL and white matter (Figure 2.1). Ectopic cell clusters are also present in the white matter at later stages of cerebellar development (P21 and adult), but the overall architecture of both the IGL and
entire cerebellum in these animals is normal. These data suggest that loss of CXCR4 results in aberrant localization of clusters of cells throughout cerebellar development.

Figure 2.1: Loss of CXCR4 results in the presence of ectopic cell clusters throughout cerebellar development.

Figure 2.1: DAPI staining of cerebella collected at E17.5, P8, P14, P21, and 8 weeks from (A) control CXCR4<sup>lox/lox</sup> or (B) Math1-Cre; CXCR4<sup>lox/lox</sup> mice. Arrows indicate ectopic cell clusters.
2.3.2 Ectopic cell clusters are granule neuron progenitors.

While analysis of cerebellar architecture in Math1-Cre; CXCR4\textsuperscript{flox/flox} mice indicated the presence of ectopic cell clusters associated with the EGL and extending into the white matter, DAPI staining alone did not provide evidence of the cell types contained within the ectopic clusters. To determine whether the ectopic clusters were composed of GNPs, we performed in situ hybridization to assess the presence of Math1 transcript (expressed specifically by GNPs in the cerebellum). As shown in Figure 2.2 A, the Math1 anti-sense probe bound specifically to GNPs within the EGL in control CXCR4\textsuperscript{flox/flox} mice. However, in Math1-Cre; CXCR4\textsuperscript{flox/flox} mice, the probe bound to both GNPs within the EGL and the ectopic cell clusters, including those associated with the EGL and those present beyond the IGL in the white matter (Figure 2.2 B). These data suggest that the ectopic clusters are primarily composed of aberrantly migrating GNPs.

![Figure 2.2](image)

**Figure 2.2:** Ectopic cell clusters express the GNP marker Math1.

**Figure 2.2:** *In situ* hybridization to assess Math1 mRNA expression in cerebella collected at P7 from (A) CXCR4\textsuperscript{flox/flox} or (B) Math1-Cre; CXCR4\textsuperscript{flox/flox} mice.
2.3.3 Premature migration of GNPs causes disruptions in Bergmann glial and Purkinje layer organization.

Given the presence of large clusters of ectopic cells between the EGL and IGL in Math1-Cre; CXCR4<sup>lox/lox</sup> mice, we questioned whether this disruption in normal GNP migration also affected the architecture of other cell types within the cerebellum. During normal cerebellar development, GNPs utilize Bergmann glia as a scaffold for migration from the EGL to IGL. The processes of Bergmann glia, whose cell bodies predominantly reside adjacent to the IGL, extend through the molecular layer and EGL to the surface of the cerebellum. To assess the effects of the presence of the ectopic cell clusters on the arrangement of Bergmann glia in Math1-Cre; CXCR4<sup>lox/lox</sup> mice, we performed immunostaining for the Bergmann glial marker BLBP. As shown in Figure 2.3A, the Bergmann glia in control CXCR4<sup>lox/lox</sup> mice are distributed in a regular pattern across the lobes of the cerebellum. However, in Math1-Cre; CXCR4<sup>lox/lox</sup> mice, this pattern is disrupted at all stages examined (Figure 2.3B). Migrating GNP clusters are associated with gaps in Bergmann glial fibers in the region surrounding the clusters at early postnatal stages. Bergmann glia are also inappropriately localized with the ectopic cell clusters in the white matter of the adult Math1-Cre; CXCR4<sup>lox/lox</sup> cerebellum. These data suggest that the aberrant migration of GNPs caused by loss of CXCR4 signaling also affects the organization of Bergmann glia.
Another important cell type for establishing the structure of the cerebellum is the Purkinje neuron. Purkinje cell bodies, which secrete the GNP mitogen Sonic hedgehog, are located immediately adjacent to the IGL, and Purkinje neurons synapse with mature granule neurons to establish a component of the circuitry of the cerebellum. To assess whether the ectopic cell clusters present in the Math1-Cre; CXCR4\textsubscript{floX/floX} mice affect the distribution of Purkinje neurons, we performed immunostaining for the Purkinje marker.
Calbindin. As shown in Figure 2.4 A, the Purkinje neuron cell bodies are properly arranged in a monolayer adjacent to the IGL in control CXCR4^{floxflox} mice. In contrast, some Purkinje cell bodies appear to be displaced by the ectopic cell clusters in the Math1-Cre; CXCR4^{floxflox} mice (Figure 2.4 B). Not only are gaps are observed in the Purkinje layer adjacent to the cell clusters at early postnatal stages, but some Purkinje neuron cell bodies are also present in association with the ectopic GNP clusters in the white matter at later postnatal stages. Together, these data suggest that the premature migration of GNP clusters resulting from loss of CXCR4 signaling in GNPs also causes disruptions in the organization of other cell types in the cerebellum.
2.3.4 CXCR4-deficient GNPs exhibit reduced Shh target gene expression, but proliferate normally.

Previous studies have indicated that in addition to regulating cellular migration, SDF-1/CXCR4 signaling may also synergize with the Shh mitogenic signal to promote proliferation in GNPs (Klein et al. 2001). Therefore, loss of CXCR4 could lead to alterations in the proliferative capacity of these cells. To determine whether Shh
responsiveness is affected in Math1-Cre; CXCR4\textsuperscript{flox/flox} mice, we performed quantitative Real-time RT-PCR to examine the expression of Shh target genes in freshly isolated GNPs from control CXCR4\textsuperscript{flox/flox} and Math1-Cre; CXCR4\textsuperscript{flox/flox} mice at postnatal day 8. As expected, cells from Math1-Cre; CXCR4\textsuperscript{flox/flox} mice display a greater than 85% reduction in CXCR4 expression compared to control cells (Figure 2.5). However, CXCR4-deficient GNPs also show a reduced expression of the Shh target genes \textit{Gli1}, \textit{N-myc}, and \textit{Cyclin D1} (Figure 2.5). This decreased expression of Shh pathway targets is consistent with the notion that SDF-1/CXCR4 signaling promotes Shh pathway activity in GNPs.

![Graph](image)

**Figure 2.5:** CXCR4-deficient GNPs exhibit reduced SHH target gene expression.

**Figure 2.5:** Real-time RT-PCR analysis of the expression levels of \textit{CXCR4} and the SHH pathway target genes \textit{Gli1}, \textit{Gli2}, \textit{Cyclin D1}, and \textit{N-myc} in GNPs isolated at P7 from CXCR4\textsuperscript{flox/flox} or Math1-Cre; CXCR4\textsuperscript{flox/flox} mice. Data are graphed as expression relative to CXCR4\textsuperscript{flox/flox}.
Because the Shh pathway is critical for driving proliferation of GNPs, we questioned whether the decreased expression of Shh targets in CXCR4-deficient GNPs was associated with impaired proliferation in Math1-Cre; CXCR4^{flox/flox} mice. To assess this, we performed immunostaining of sections from control CXCR4^{flox/flox} and Math1-Cre; CXCR4^{flox/flox} mice for the proliferation marker Ki67 and the granule neuron differentiation marker NeuN. As shown in Figure 2.6 A, GNPs within in the EGL of Math1-Cre; CXCR4^{flox/flox} mice display a proliferation pattern similar to that of the control. Surprisingly, the ectopic migrating clusters also maintain expression of Ki67 as they migrate out of the mitogenic niche and into and through the region of differentiated granule neurons in the IGL. Furthermore, these ectopic cells do not express the differentiation marker NeuN during migration in these clusters. These data suggest that CXCR4 signaling is not required for GNP proliferation in vivo and that the aberrantly migrating cells maintain their identity as proliferating progenitors despite premature migration away from the mitogenic niche.
Figure 2.6: CXCR4-deficient GNPs proliferate normally *in vitro* and *in vivo*.

**Figure 2.6:** (A) Cerebella collected at P8 from CXCR4\textsuperscript{lox/lox} or Math1-Cre; CXCR4\textsuperscript{lox/lox} mice were immunostained with antibodies against the proliferation marker Ki67 (green), neuronal marker NeuN (red), and DAPI (blue). **(B)** GNPs were isolated from P7 cerebella from CXCR4\textsuperscript{lox/lox} or Math1-Cre; CXCR4\textsuperscript{lox/lox} mice, plated, and immediately pulsed with \(^3\)H-Td in culture for 4 hours or 12 hours. Data represent average \(^3\)H-Td incorporation from triplicate wells for each condition. **(C)** GNPs were isolated from P7 cerebella from CXCR4\textsuperscript{lox/lox} or Math1-Cre; CXCR4\textsuperscript{lox/lox} mice, plated on coverslips, and cultured in the presence or absence of 3 \(\mu\)g/ml Shh for 48 hours. Coverslips were stained with an anti-Ki67 antibody (red) and DAPI (blue).
To quantitatively investigate the proliferative capacity of these cells, we isolated GNPs from control CXCR4^{floxed} and Math1-Cre; CXCR4^{floxed} animals at P7, immediately pulsed the cells with ^3H-thymidine, and measured incorporation 4 hours or 16 hours later. CXCR4-deficient GNPs displayed levels of thymidine incorporation that were similar to the control, again suggesting that these cells proliferate normally (Figure 2.6 B). To assess whether Math1-Cre; CXCR4^{floxed} GNPs respond appropriately to Shh, we cultured control and mutant cells in the presence or absence of Shh for 2 days and assessed proliferation by BrdU uptake (data not shown) or Ki67 immunostaining (Figure 2.6 C). Again, the CXCR4-deficient GNPs showed no defect in proliferation after Shh treatment, suggesting that cells lacking CXCR4 are capable of an appropriate proliferative response to Shh.

2.3.5 Ectopic GNPs differentiate appropriately

Migration of GNPs away from the EGL is normally coupled with exit from the cell cycle and differentiation (Choi et al. 2005). The observation that prematurely migrating CXCR4-deficient GNPs continue to proliferate upon exit from the EGL raises the possibility that the aberrant localization of these cells might allow them to escape their normal differentiation program. To determine whether GNP differentiation is affected in Math1-Cre; CXCR4^{floxed} mice, we performed immunostaining for the proliferation marker Ki67, the mature neuronal marker NeuN, and the mature granule neuron marker GABARA6 at later stages of cerebellar development, including P14, P21
and adult. While abnormal clusters of cells are present at all stages examined, these clusters (including those that ultimately reside in the white matter of the adult cerebellum) differentiate with timing similar to wild-type GNPs (Figure 2.7 A and B). The ectopic clusters that migrate into the white matter, an unusual microenvironment for GNP differentiation, ultimately express NeuN and GABARA6, suggesting that commitment to the granule cell lineage and ultimate survival is not affected by loss of CXCR4 (Figure 2.7 C).
Figure 2.7: Loss of CXCR4 does not affect ultimate differentiation into granule neurons.

Figure 2.7: Cerebella were collected at P21 or 8 weeks from CXCR4\textsuperscript{lox/lox} (A) or Math1-Cre; CXCR4\textsuperscript{lox/lox} (B) mice and immunostained with an antibody against the mature neuronal marker NeuN (red). Arrows indicate differentiated ectopic clusters in Math1-Cre: CXCR4\textsuperscript{lox/lox} cerebella. (C-E) Inset for higher magnification of ectopic clusters in white matter of P21 cerebellum from Math1-Cre; CXCR4\textsuperscript{lox/lox} mice. Immunostaining for (C) Neuronal marker NeuN (red), (D) Granule neuron marker GABRA6 (green), and (E) overlay of NeuN + GABRA6.

2.3.6 CXCR4 is not required after postnatal day 4 for appropriate GNP migration

In Math1-Cre; CXCR4\textsuperscript{lox/lox} mice, CXCR4 deletion begins at approximately E13 and persists throughout development. Our data thus far suggest that this deletion has
consequences on the postnatal migration of GNPs, but it is unclear whether this consequence is a result of continued dependence on CXCR4 or a requirement for CXCR4 signaling during a specific period of development. To address the temporal requirement for CXCR4 in GNPs, we created Math1-CreER; CXCR4^{floxed/floxed} animals to induce CXCR4 deletion upon tamoxifen administration at postnatal days P1, P2, P4, and P5. Tamoxifen administration at P1 or P2 led to an approximately 70% reduction in CXCR4 expression at P7 and premature GNP migration similar to that observed in Math1-Cre; CXCR4^{floxed/floxed} animals (Figure 2.8 A and data not shown). In contrast, while tamoxifen administration at P4 or P5 led to a similar reduction in CXCR4 expression, premature GNP migration was not observed upon examination at P7, P10, or P14 (Figure 2.8 B and data not shown). Together, these data suggest that a subset of GNPs require CXCR4 signaling throughout embryonic development and for the first 2-3 days after birth to establish proper radial migration throughout postnatal development.
Figure 2.8: CXCR4 deletion after post-natal day 4 does not cause aberrant migration.

Figure 2.8: (A) Math1-CreER; CXCR4^{fl/+} pups were treated with tamoxifen at P1, and cerebella were collected at P14. Sections were immunostained for the proliferation marker Ki67 (green), neuronal marker NeuN (red), and DAPI. (B) Math1-CreER; CXCR4^{fl/+} pups were treated with tamoxifen at P5, and cerebella were collected at P14. Sections were immunostained for the proliferation marker Ki67 (green), neuronal marker NeuN (red), and DAPI. CXCR4 deletion at P5 does not result in ectopic proliferating cell clusters.

2.3.7 Loss of CXCR4 does not impair tumor formation

Previous studies have indicated that CXCR4 signaling may promote tumor growth in SHH-associated medulloblastoma (Rubin et al. 2003; Yang et al. 2007; Sengupta et al. 2012). Patched mutant mice have been used to model this subtype of the human disease (Goodrich et al. 1997). Tumors that develop in these mice as a result of
constitutive activation of the Shh pathway are thought to arise from GNPs that fail to migrate out of the EGL and instead remain on the surface of the cerebellum in close association with the meninges (Oliver et al. 2005). Given the proposed role for CXCR4 signaling in both synergizing with Shh to promote proliferation and in regulating GNP migration, we questioned whether loss of CXCR4 signaling would affect tumor formation in patched mutant mice. To do this, we generated control Math1-Cre; Ptc^lox/lox mice and CXCR4-deficient Math1-Cre; Ptc^lox/lox, CXCR4^lox/lox mice and monitored the animals for symptoms of tumors. As shown in Figure 2.9, loss of CXCR4 signaling does not affect overall survival, suggesting that CXCR4 is not required for tumorigenesis in the patched mutant mouse model of medulloblastoma.

Figure 2.9: Loss of CXCR4 does not affect tumor formation in patched mutant mice.

Figure 2.9: Kaplan-Meier analysis of survival upon deletion of patched (Math1-Cre; CXCR4^+/+;Ptc^flo/flo) or deletion of patched and CXCR4 (Math1-Cre; CXCR4^flo/flo;Ptc^flo/flo). No significant difference between the curves was observed.
2.4 Discussion

SDF-1/CXCR4 signaling has been shown to contribute to appropriate migration of GNPs during embryonic development, but the role of CXCR4 in postnatal development has remained unknown. Here, we have utilized Math1-Cre; CXCR4$^{\text{flox/flox}}$ mice to delete CXCR4 specifically in GNPs (avoiding the perinatal lethality of conventional CXCR4$^{-/-}$ mice) and assess the contribution of CXCR4 to postnatal cerebellar development and the ultimate fate of affected cells. We show that the conditional knockout mice display aberrant migration of a subset of GNPs that persists throughout GNP maturation, but the ectopic cells are ultimately capable of appropriate differentiation regardless of their location. Furthermore, we show that CXCR4 deficiency leads to a reduction in Shh pathway activity in GNPs, but these cells maintain their full proliferative capacity both in vitro and in vivo. Together, our data indicate that CXCR4 signaling is dispensable for proliferation and differentiation, but is required for appropriate radial migration of a subset of GNPs throughout cerebellar development.

Previous studies using conventional CXCR4$^{-/-}$ or SDF-1$^{-/-}$ knockout mice have demonstrated that loss of SDF-1/CXCR4 signaling causes premature migration of embryonic GNPs from the EGL into the cerebellar cortex (Ma et al. 1998; Zou et al. 1998). Our data, using conditional deletion of CXCR4 specifically in GNPs, are consistent with the previous reports; Math1-Cre; CXCR4$^{\text{flox/flox}}$ mice also display aberrant migration of clusters of GNPs “streaming” from the EGL into the IGL and white matter, coincident
with displacement of Bergmann glial fibers and Purkinje cell bodies in the regions of the ectopic GNP clusters. Our data confirm that this phenotype is a direct consequence of loss of CXCR4 specifically in GNPs, rather than an indirect effect on GNPs mediated by disruptions of CXCR4 signaling in other cell types.

Given the proposed role for the SDF-1/CXCR4 interaction as responsible for maintaining GNPs in the EGL until the appropriate time to differentiate, the finding that only a subset of GNPs are affected by loss of CXCR4 is surprising. One possible explanation for this could be that CXCR4 is not efficiently deleted in the unaffected GNPs; however, our analysis of the levels of CXCR4 transcript expression in Math1-Cre; CXCR4<sup>fl/fl</sup> GNP<sup>s</sup> indicated a greater than 85% decrease in CXCR4 expression, which likely could not be achieved with deletion in only a small subset of cells. Furthermore, the conventional CXCR4<sup>−/−</sup> mice, in which CXCR4 expression is lost in all cells, also display this phenotype. Another possible explanation for the absence of an effect on the majority of GNPs in the EGL could be compensation through an alternative receptor. CXCR7 has recently been identified as an alternate receptor for SDF-1 (Burns et al. 2006). However, CXCR7 is not normally expressed in GNPs (Schonemeier et al. 2008), and our analysis of CXCR7 expression in Math1-Cre; CXCR4<sup>fl/fl</sup> mice indicated that expression of this receptor is not upregulated in CXCR4-deficient GNPs (data not shown). Furthermore, much of the EGL remains similarly unaffected in SDF-1<sup>−/−</sup> mice (Ma et al.)
1998), suggesting that signals mediated by SDF-1 (whether through CXCR4 or CXCR7) are not required for appropriate migration of these unaffected GNPs.

Another possible explanation for why only a subset of GNPs are affected by loss of CXCR4 signaling could be heterogeneity among GNPs in the EGL with regard to the signals required for the appropriate timing of differentiation and migration. Some cells within the EGL may be dependent upon alternative migration signals, such as Plexin-B2. Plexin-B2 has also been shown to regulate GNP radial migration, and mice in which Plexin-B2 or its ligands Sema4C and Sema4G have been inactivated through a gene-trap strategy also display premature migration of dispersed individual GNPs, rather than in cell clusters (Friedel et al. 2007; Maier et al. 2011). The premature migration effects of both CXCR4 and PlexinB2 deficiency highlight the possibility that distinct populations of GNPs within the EGL may be dependent on one or the other, or both, of these signaling pathways for appropriate migration.

Potential heterogeneity in GNPs may also be reflected in the timing of dependency on SDF-1/CXCR4 signaling. Because our studies of Math1-Cre; CXCR4<sup>flox/flox</sup> mice and previous studies of CXCR4<sup>−/−</sup> and SDF-1<sup>−/−</sup> mice have assessed the effects of loss of CXCR4 early in development, it is clear that signaling through this pathway is required at embryonic stages for the appropriate regulation of GNP migration. However, our analysis of CXCR4 deletion at postnatal stages (using Math1-CreER; CXCR4<sup>flox/flox</sup> mice) indicated that after postnatal day 4, GNPs are no longer dependent
on CXCR4 signaling for preventing premature migration. These data are consistent with previous analyses demonstrating that the capacity of GNPs to respond to SDF-1 changes over time; GNPs isolated from embryonic cerebella demonstrate clear chemoattraction towards an SDF-1 gradient in vitro, whereas GNPs isolated from postnatal cerebella display greatly diminished chemoattraction (Zhu et al. 2002; Zhu et al. 2004). Our data similarly suggest that SDF-1/CXCR4 signaling is no longer required for appropriate migration after P4. Interestingly, although late postnatal deletion of CXCR4 does not affect GNP migration, the premature migration phenotype initiated by embryonic CXCR4 deletion persists throughout postnatal cerebellar development, suggesting that early interruption in this signaling can continue to affect the cells beyond the stage at which they are dependent upon SDF-1/CXCR4. Further studies will be required to understand the mechanisms responsible for this prolonged effect.

In addition to regulating GNP migration, synergy between SDF-1 and Shh signaling has been proposed to promote GNP proliferation. Previous studies have demonstrated enhanced proliferation in vitro upon treatment of postnatal GNP with the combination of SDF-1 plus Shh, compared to Shh alone (Klein et al. 2001). In addition, Shh and SDF-1 signaling have been shown to reciprocally regulate each other, such that activation of the Shh pathway induces increased expression of CXCR4, while increased CXCR4 signaling promotes Shh pathway activity (Sengupta et al. 2012). Our data demonstrating reduced Shh pathway activation (measured by expression of the Shh...
pathway targets Gli1, Gli2, N-myc, and Cyclin D1) in Math1-Cre; CXCR4^{floxed} mice are consistent with these reports. However, we did not observe any impairment of proliferation in Math1-Cre; CXCR4^{floxed} GNPs in response to Shh in vitro or in vivo. It is important to note that the synergy observed between SDF-1 and Shh in previous studies was modest and required low concentrations of Shh, with only an approximately 1.5-fold increase in proliferation upon treatment with SDF-1 plus Shh compared to Shh or SDF-1 alone (Klein et al. 2001). While these previous studies and our expression data suggest that SDF-1 can enhance Shh signaling, our analysis suggests that synergy with SDF-1/CXCR4 signaling is not required for GNP proliferation.

The ability of the ectopic clusters of CXCR4-deficient cells to proliferate outside of the EGL is also surprising. Previous reports have defined the EGL as a “mitogenic niche” for GNPs, and exit from this niche is thought to cause differentiation (Choi et al. 2005). Our data indicating that CXCR4-deficient GNPs can continue to proliferate outside of the EGL highlight the question of whether the timing of proliferation is dependent upon extracellular signals vs. an intrinsic proliferation/differentiation program. It is possible that the ectopic GNPs can recruit (or physically push/drag) the extracellular signals or supporting cell types required for continued proliferation along with the ectopic clusters. For example, Purkinje cell bodies were observed in association with the ectopic clusters migrating through the IGL and in the white matter in Math1-Cre; CXCR4^{floxed} mice. Because Purkinje cells are thought to be the source of the Shh
mitogen (Wechsler-Reya and Scott 1999), this displacement of Purkinje cells with the ectopic cells may have enabled continued exposure of the aberrantly migrating GNPs to Shh, thus allowing the establishment of a traveling “micro-mitogenic niche” and continued proliferation outside of the EGL.

An alternative explanation for the ability to proliferate outside of the EGL could be that the cells harbor an intrinsic program for proliferation and differentiation. Although the ectopic cells remained associated with Purkinje cells, they ultimately differentiated into granule neurons (regardless of their location) with timing similar to their wild-type counterparts, suggesting that the signals that allowed the continued proliferation in aberrant locations were not sufficient to prevent cell cycle exit and differentiation. Even in wild-type animals, GNPs also are capable of exiting the cell cycle and initiating differentiation within the EGL, still in proximity to Purkinje cells and Shh ligand. Together, these data highlight the complexity involved in the regulation of GNPs development, and further studies will be required to elucidate the mechanisms that define the timing of proliferation vs. differentiation.

Inhibition of CXCR4 signaling, using the small molecule CXCR4 antagonist AMD-3100, has been shown to inhibit proliferation of cells from the SHH-associated subtype of medulloblastoma in both mouse models and xenografts of human cell lines (Rubin et al. 2003; Yang et al. 2007; Sengupta et al. 2012). These data are in contrast to our results in Math1-Cre; Ptc<sup>flox/flox</sup>; CXCR4<sup>flox/flox</sup> mice, which developed tumors with the
same latency as Math1-Cre; Ptc<sup>flox/flox</sup> (CXCR4 wild-type) mice. One difference between our studies and previous studies is that other studies have examined the effects of acute inhibition of CXCR4 signaling within already established tumors (or tumor cell lines), whereas our study assessed the effects of loss of CXCR4 signaling on the formation of tumors. In addition, it is unclear in the previous studies what cell types (the tumor cells themselves vs. surrounding tissue) were affected by CXCR4 inhibition, while our study assessed the effects of eliminating CXCR4 signaling in GNPs, the cell type thought to give rise to SHH-associated tumors. Our data suggest that GNPs are able to overcome loss of CXCR4 signaling for continued proliferation and tumor formation, whereas tumors that develop with competent CXCR4 signaling may remain dependent on this signal for continued growth. However, the ability of the tumor cells/cell of origin to escape dependency on CXCR4 signaling may suggest that targeting CXCR4 alone may not be an effective approach for clinical treatment of medulloblastoma, and could instead be considered in the context of combination therapy with treatment with Hedgehog antagonists.

Together, our data suggest that while other factors are likely involved in regulating migration, CXCR4 clearly plays an important role in a subset of GNPs. In addition, although CXCR4 can enhance GNP proliferation, signaling through this pathway is not absolutely required during development or tumor initiation. Future
studies will be required to understand how CXCR4 signaling relates to and interacts with other pathways to regulate the proper development of the cerebellum.
3. Biochemical Identification of CD15-associated molecules and Immunological Targeting of CD15+ Tumor-Propagating Cells

3.1 Introduction

Tumor-propagating cells (TPCs), also known as cancer stem cells, have been defined as the cells within a tumor that are capable of regenerating the tumor upon transplantation into a naïve host. TPCs have been identified in a variety of tumor types, including those of the brain, breast, prostate, colon, pancreas, liver, lung, and skin, among others (Al-Hajj et al. 2003) (Singh et al. 2004) (O’Brien et al. 2007) (Li et al. 2007) (Xin et al. 2005) (Bertolini et al. 2009) (Yang et al. 2008a) (Schober and Fuchs 2011). The ability of TPCs to regenerate tumors has led to the notion that these cells are responsible for tumor resistance and recurrence after therapy. Indeed, TPCs have been shown to display resistance to both chemotherapy and radiation (Alison et al. 2012) (Bao et al. 2006) (Liu et al. 2006). Given this capacity for evading standard therapies and regenerating tumors, identification of therapeutic approaches to target and eliminate these cells could substantially improve patient outcomes.

We recently identified a population of TPCs in patched heterozygous mice, a widely studied mouse model of SHH-associated MB (Read et al. 2009). These cells, which can be identified based on their expression of the cell surface carbohydrate antigen CD15/SSEA-1, are uniquely capable of propagating tumors following transplantation. When CD15+ cells are transplanted into the cerebella of naïve mice,
100% of recipients develop tumors, whereas CD15- cells never generate tumors. Expression profiling revealed that CD15+ cells display decreased expression of genes associated with differentiation and elevated expression of genes associated with proliferation. CD15 is also found in a subset of human MBs, and patients whose tumors express high levels of a CD15-associated gene signature have a poorer prognosis. However, important questions remain, including (1) the identity of the molecule that carries this antigen in patched tumor cells and (2) whether these tumor-propagating cells are required for continued tumor growth.

As a trisaccharide carbohydrate antigen, the CD15 epitope (also known as SSEA-1 or Lewis X) is comprised of fucose, galactose, and acetylglucosamine (Figure 3.1) (Gooi et al. 1981). CD15 has been shown to be expressed on multiple cell types, including embryonic stem cells, CNS stem and progenitor cells, and terminally differentiated myeloid cells (Capela and Temple 2002; Yanagisawa et al. 2005) (Stocks et al. 1990) (Warren et al. 1996) (Muramatsu 1988) (Muramatsu and Muramatsu 2004). Both proteins and lipids can carry the CD15 epitope, but these carriers can differ in different cell types, and CD15 expression does not necessarily correlate with expression of a single protein or lipid molecule (Hennen et al. 2011) (Yanagisawa et al. 2005) (Allendoerfer et al. 1995) (Capela and Temple 2006). While expression of CD15 marks TPCs in SHH-associated MB, the function of the CD15 epitope itself or its carrier molecules remains unknown. Identification of the CD15 carrier in patched mutant
tumors would provide insight into the role of this molecule in tumorigenesis. Here, we have employed a biochemical approach to identify the CD15 carrier molecule in these tumors, with the aim of understanding the role of the CD15 molecule in tumor propagation.

Figure 3.1: Structure of the CD15 epitope.

**Figure 3.1:** The CD15 trisaccharide epitope is composed of fucose, galactose, and acetylglucosamine (Galβ1-4(Fucα1-3)GlcNAc). The epitope can be present on O-glycans or N-glycans and attached to either proteins or lipids.

In addition to identification of the CD15-associated carrier molecule, it is also important to identify approaches for targeting these cells. While TPCs have been shown to be capable of regenerating tumors upon transplantation, it remains unknown whether targeting/elimination of these cells in an endogenous tumor can indeed cause tumor regression and/or prevent tumor recurrence. To address this question, we have employed an immunotoxin approach, in which the ribosome inactivating protein Saporin is conjugated to the CD15 antibody used to identify the TPCs, to specifically
eliminate these cells and determine their necessity for continued tumor progression. If successful, this approach could enable assessment of the role of TPCs in tumor development and potentially provide novel approaches for MB therapy.

### 3.2 Methods

#### 3.2.1 Methods for biochemical identification of CD15 carrier molecule

##### 3.2.1.1 Cell lines and mice

The F9 teratocarcinoma cell line and Ba/F3 pro-B cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). F9 cells were cultured in DMEM media with 10% FBS and 1% Penicillin/Streptomycin (Invitrogen). Ba/F3 cells were cultured in RPMI-1640 media with 10% FBS, 1% Penicillin/Streptomycin (all from Invitrogen), and 10 ng/ml IL-3 (PeproTech, Rocky Hill, NJ).

Germline patched heterozygous mutant mice (Goodrich et al. 1996) were maintained by breeding with 129X1/SvJ or C57BL/6 mice from The Jackson Laboratory (Bar Harbor, ME). Conditional Math1-CreER; Ptc\textsuperscript{flox/flox} mice were treated with 0.8 mg of tamoxifen (T5648, Sigma, St. Louis, MI) in 40 μl of corn oil at post-natal day 4 to generate tumors 10-16 weeks later (Yang et al. 2008b).

##### 3.2.1.2 Isolation of patched mutant tumor cells

Tumors were obtained from germline patched heterozygous or conditional Math1-CreER; Ptc\textsuperscript{flox/flox} mice. The tumors were minced using scissors, digested in a papain solution for 30 minutes at 37°C to obtain a single-cell suspension, then
centrifuged through a 35%-65% Percoll gradient. Cells from the 35%-65% interface were collected, frozen, and stored at -80°C until lysis for immunoprecipitation experiments, or cultured in NB-B27 (Neurobasal with 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin/streptomycin, and B27 supplement) plus 1% FBS (Invitrogen) for immunotoxin experiments. For culture, the cells were plated on poly-D-lysine-coated plates.

3.2.1.3 Generation of anti-CD15-agarose

The CD15 antibody (clone MMA, BD Biosciences) or control mouse IgM was linked to agarose using a Pierce AminoLink Plus Immobilization kit following the manufacturer’s instructions (Pierce, Rockford, IL). Briefly, 200 μg of antibody or IgM was incubated with 400 μl of 4% beaded agarose slurry in Coupling Buffer for 4 hours and crosslinked with sodium cyanoborohydride. The mixture was then washed, incubated with Quenching Buffer, washed again, and stored at 4°C until use.

3.2.1.4 Immunoprecipitation and Western blot

To immunoprecipitate CD15-associated proteins from patched mutant tumors, cells were lysed in RIPA buffer (Millipore, Billerica, MA) containing 1 mM sodium orthovanadate, 2 mM sodium fluoride (both from Sigma, St. Louis, MO), and Complete, Mini, EDTA-free protease inhibitor tablets (Roche Applied Science, Indianapolis, IN). Proteins (1000-15000 μg) were incubated in the presence of IgM-agarose or anti-CD15-agarose on ice for 2 hours. The samples were then washed and eluted with sample
buffer containing 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% Glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, and 0.02% Bromophenol blue. The eluted proteins were then resolved by SDS-PAGE on 4-20% Tris-Glycine gels and transferred to nitrocellulose membranes (Invitrogen), which were then probed with an unconjugated anti-CD15 antibody (clone MMA 1:100, BD Biosciences), followed by a goat anti-mouse IgM antibody conjugated to IRdye 680 (Rockland, Gilbertsville, PA). Proteins were detected using the Odyssey imaging system (LI-COR, Lincoln, NE).

3.2.1.5 Cross-linking of antibody to cells
To try to improve the binding of the antibody to the CD15 epitope, we incubated cells with antibody and crosslinked the antibody to the antigen prior to lysis. For these experiments, biotin-conjugated CD15 antibody and control IgM was generated using the Pierce EZ-Link Sulfo-NHS-Biotinylation Kit following the manufacturer’s instructions. Cells (~10 million) were then incubated in the presence of control IgM-biotin or anti-CD15-biotin for 1 hour on ice. The cells were then washed and incubated with the crosslinking agent Disuccinimidyl suberate (DSS; Pierce) (prepared according to manufacturer’s instructions) for 30 minutes at room temperature. The reaction was then quenched with the addition of 20 mM Tris for 15 minutes at room temperature. The cells were then lysed, and immunoprecipitation (using Streptavidin-agarose) and Western blot were performed as described above.
3.2.1.6 Removal of CD15-associated proteins and lipids from cell surface

To test whether CD15 is expressed on protein vs. lipid in patched mutant tumors, cells were isolated as described above and incubated with either enzymes to remove proteins or methanol to remove lipids. To remove proteins, cells were treated with papain (10 U/ml; Worthington Biochemicals), pronase (0.5 mg/ml; Sigma), or trypsin (1 mg/ml; Sigma) in PBS for 1 hour at 37°C. To remove lipids, cells were incubated with 50% or 100% methanol (Sigma) for 20 minutes at room temperature. The cells were then washed with PBS, and CD15 expression was assessed by flow cytometry.

3.2.1.7 Flow cytometry to assess CD15 expression

To determine CD15 expression after removal of proteins or lipids, cells were stained with control mouse IgM or anti-CD15 (clone MMA, BD Biosciences) antibodies, followed by anti-mouse IgM-phycoerythrin (PE) (Jackson ImmunoResearch, West Grove, PA). The cells were then analyzed on a FACSVantage flow cytometer (BD Biosciences).

3.2.2 Methods for immunotoxin-based targeting of CD15+ cells

3.2.2.1 Reagents

All immunotoxin reagents, including the secondary anti-IgM-saporin (anti-IgM-SAP) and the directly conjugated control IgM-SAP and anti-CD15-SAP were obtained from Advanced Targeting Systems (ATS, San Diego, CA). The directly conjugated
CD15-SAP immunotoxin was generated by ATS using the anti-CD15 clone MMA antibody produced by QED Bioscience, Inc. (San Diego, CA).

3.2.2.2 Proliferation of cells cultured in the presence of immunotoxin

To assess effects of the immunotoxins on proliferation, tumor cells from patched mutant mice were isolated as described above and plated in 96-well plates at a density of 0.2 million cells per well. Cells were cultured in the presence of the indicated concentrations of immunotoxins for 48 hours in triplicate wells, then pulsed with [methyl-³H]thymidine (Amersham/GE Healthcare, Piscataway, NJ) and cultured for an additional 16–18 hours. Cells were harvested onto filters using a Mach IIIM Manual Harvester 96 (Tomtec, Hamden, CT), and incorporated radioactivity was quantified by liquid scintillation spectrophotometry on a Wallac MicroBeta scintillation counter (PerkinElmer, Waltham, MA).

3.3 Results

3.3.1 Biochemical identification of CD15 carrier molecule

3.3.1.1 Immunoprecipitation/Western blot approach can identify a CD15-associated molecule in F9 cells

Our previous studies indicated that cells expressing the CD15 carbohydrate epitope are capable of propagating patched mutant tumors, but the identity of the carrier molecule for CD15 in these tumors remains unknown. To identify CD15-associated molecules, we planned to perform immunoprecipitation using the CD15 antibody, followed by mass spectrometry for protein identification.
To validate that this approach could work, we first assessed whether immunoprecipitation using the CD15 antibody allows detection of a CD15-associated protein in F9 teratocarcinoma cells. This cell line was chosen to validate the approach because FACS analysis indicates that greater than 95% of F9 cells display CD15 on their cell surface (data not shown), and previous studies have indicated that the CD15 epitope is associated with a high molecular weight protein in these cells (Childs et al. 1983). As shown in Figure 3.2, incubation with either the CD15 antibody followed by precipitation with the secondary anti-IgM-agarose or with the CD15 antibody directly conjugated to agarose led to Western blot detection of a very high molecular weight protein. These high molecular weight bands were not detected upon incubation with control IgM or IgM-agarose. These data suggest that the CD15 antibody and immunoprecipitation approach can be used to isolate CD15-associated proteins from cells expressing the epitope.
Figure 3.2: CD15 antibody can immunoprecipitate high molecular weight protein in F9 teratocarcinoma cells.

**Figure 3.2:** Lysate from F9 cells was immunoprecipitated using the indicated antibodies, and Western blot was performed using an anti-CD15 antibody (clone MMA). Arrow indicates high molecular weight CD15-associated protein.

3.3.1.2 Immunoprecipitation/Western blot approaches fail to identify a CD15-associated protein in patched mutant tumor cells

To identify CD15-associated proteins in patched mutant tumor cells, we performed immunoprecipitation by incubating lysate with agarose-conjugated control IgM or CD15 antibody. However, as shown in Figure 3.3, Western blot analysis using
the CD15 antibody failed to identify any protein specifically immunoprecipitated by anti-CD15-agarose. Compared to anti-CD15-agarose alone (not incubated with lysate), no additional protein bands were detected after incubation of anti-CD15-agarose with lysate. Furthermore, many fewer bands were detected in the anti-CD15-agarose (with lysate) sample than in the IgM-agarose (with lysate) sample. One possible explanation for not detecting CD15-associated proteins could be that the CD15 antibody is a poor reagent for Western blot. However, protein detection by Western blot using other CD15 antibody clones or lectins, direct staining by glycoprotein (periodic acid-Schiff), Comassie, or silver staining of the gel, or $^{35}$S protein labeling also failed to demonstrate any specific CD15-associated protein bands (data not shown). These data suggest that a "standard" immunoprecipitation approach may not allow for isolation of CD15 carrier proteins in patched mutant tumor cells.
Figure 3.3: CD15 antibody does not immunoprecipitate an associated protein from patched mutant tumor cells.

Figure 3.3: Lysate from patched mutant tumor cells was immunoprecipitated using the indicated antibodies, and Western blot was performed using an anti-CD15 antibody (clone MMA). No specific CD15-associated protein bands were detected.

One explanation for the inability of the CD15 antibody to precipitate CD15-associated proteins may be that, as an IgM antibody, the affinity of the antibody for the epitope is too low to precipitate sufficient quantities of protein for detection. To address this possibility, we employed an immunoprecipitation strategy that was based on
antibody binding to the epitope in intact cells. In this approach, *patched* mutant tumor cells were incubated with biotin-conjugated control IgM or CD15 antibody, the antibodies were cross-linked to the protein using Disuccinimidyld suberate (DSS, an amine-to-amine crosslinker), the cells were lysed, and immunoprecipitation was performed on the lysate using streptavidin-agarose. As shown in Figure 3.4 A, immunoprecipitation using this approach followed by Western blot with the CD15 antibody yielded detection of multiple high molecular weight protein bands in samples that had been incubated with anti-CD15-biotin and crosslinked with DSS (but not in samples form cells that had not been treated with DSS or in samples without lysate containing only the antibodies). However, Western blot using only the anti-IgM secondary antibody also detected these bands, suggesting that this protein was likely non-specific, rather than the CD15 carrier protein (Figure 3.4 B). Furthermore, this band could not be reproducibly detected upon repeat of the experiment using cells from additional *patched* mutant tumors. Together, these data suggest that immunoprecipitation strategies using the CD15 antibody MMA clone do not isolate CD15 carrier proteins in *patched* mutant tumor cells.
Figure 3.4: Cross-linking of CD15 antibody to CD15 epitope on cell surface does not enable identification of CD15-associated protein.

Figure 3.4: Cells from patched mutant tumor cells were incubated with the indicated antibodies and DSS crosslinker prior to lysis. Immunoprecipitation was performed using Streptavidin-agarose, and Western blot was performed using either (A) an anti-CD15 antibody (clone MMA) or (B) the secondary antibody anti-IgM-IR680 alone. The bands detected upon incubation of cells with anti-CD15-biotin plus the DSS crosslinker by anti-CD15 Western blot were also detected by Western blot with secondary antibody alone (indicated by brackets in both cases).
3.3.1.3 CD15 may be associated with both protein and lipid molecules in *patched* mutant tumor cells

Another explanation for our inability to isolate CD15-associated proteins in *patched* mutant tumor cells is that the CD15 epitope could potentially be expressed on lipids, not proteins, in these cells. To address this possibility, we examined CD15 expression by FACS analysis after treating cells with reagents to remove proteins (such as the enzymes pronase or trypsin) or lipids (such as methanol) from the cell surface. As shown in Figure 3.5 A and B, treatment of a representative *patched* mutant tumor with pronase or trypsin caused CD15 expression to decrease from approximately 43% to approximately 23%, while treatment with methanol caused expression to decrease to approximately 20%. Treatment of these cells with the combination of pronase plus methanol almost completely eliminated binding of the CD15 epitope to the cells. Together, these data suggest that the CD15 epitope may be expressed on both proteins and lipids in *patched* mutant tumor cells.
Figure 3.5: CD15 epitope detection is affected by removal of proteins and lipids from the cell surface.

Figure 3.5: Freshly isolated *patched* mutant tumor cells were treated with pronase or trypsin (to cleave cell surface proteins) or methanol (to remove lipids), then CD15 expression was assessed by flow cytometry. (A) Histograms of control mIgM or CD15 expression in the different conditions. (B) Graph of percentage of cells within the “positive” gate for each condition.
3.3.2 Immunotoxin-mediated targeting of CD15+ cells

3.3.2.1 CD15 antibody plus Saporin-conjugated secondary antibody can effectively inhibit proliferation

While identification of the CD15 carrier molecules in patched mutant tumor cells would allow exploration of the function of the CD15 epitope and carrier, directly targeting this (these) molecules in a therapeutic setting may not be easily achievable. To directly target the CD15-expressing TPCs, regardless of the identity of the carrier molecule, we employed an immunotoxin approach using Saporin-conjugated antibodies. Saporin is a ribosome-inactivating protein isolated from the soapwort plant (Saponaria officinalis) that interferes with protein translation, ultimately causing cell death (Stirpe et al. 1983) (Lappi et al. 1985). When a Saporin-conjugated antibody binds to the cell surface, the antibody-toxin conjugate is internalized, the disulfide bond linking the toxin to the antibody is cleaved, and the toxin is released, binds to ribosomes, and causes cell death (Mathew and Verma 2009).

To determine whether this Saporin-conjugated immunotoxin approach can kill CD15-expressing tumor cells, we treated F9 cells or patched mutant tumor cells with control IgM or CD15 antibody either with or without a secondary anti-IgM-Saporin (mZAP) secondary antibody. As shown in Figure 3.6 A, treatment with the control IgM or CD15 antibody alone or with IgM plus the secondary mZAP did not affect proliferation in F9 cells, but treatment with the CD15 antibody plus mZAP significantly inhibited proliferation in a dose-dependent manner. Furthermore, treatment of patched
mutant tumor cells with the CD15 antibody plus mZAP also substantially inhibited proliferation (Figure 3.6 B). These data suggest that antibody-conjugated Saporin can effectively block the growth of CD15-expressing cells.

Figure 3.6: Treatment of F9 cells or patched mutant tumor cells with CD15 primary antibody plus saporin-conjugated secondary antibody inhibits proliferation.

Figure 3.6: F9 cells (A) or patched mutant tumor cells (B) were cultured in the presence of the indicated antibodies for 48 hours, then pulsed with ^3H-Td and cultured for an additional 16 hours. Data represent average ^3H-Td incorporation from triplicate wells for each condition.
3.3.2.2 Directly conjugated CD15-SAP immunotoxin specifically inhibits proliferation of F9 cells

Although the inhibition of proliferation observed upon treatment of cells with the combination of the CD15 primary antibody plus the secondary mZAP provides encouraging proof-of-principle for the immunotoxin approach, the use of separate primary and secondary antibodies is not ideal for the goal of employing this approach in in vivo models or ultimately in a therapeutic setting. Therefore, we generated a Saporin-conjugated CD15 antibody (CD15-SAP) to directly target the CD15-expressing cells with a single antibody. To assess whether this directly conjugated immunotoxin could also effectively inhibit the growth of CD15-expressing cells, we treated F9 cells with directly-conjugated IgM-SAP or CD15-SAP. As shown in Figure 3.7 A, treatment with CD15-SAP caused a significant dose-dependent inhibition of proliferation and reduction in the number of viable cells. To assess the specificity of the CD15-SAP toxin, we also treated Ba/F3 cells with IgM-SAP or CD15-SAP. This cell line was chosen because fewer than 2% of Ba/F3 cells express CD15 (data not shown); therefore, the CD15 immunotoxin should not be able to bind to the cells or affect viability. Indeed, CD15-SAP did not significantly affect proliferation of Ba/F3 cells at any concentration (Figure 3.7 B). Together, these data suggest that the directly conjugated CD15-SAP immunotoxin can effectively and specifically inhibit the proliferation of CD15-expressing cells.
3.3.2.3 Both control IgM-SAP and CD15-SAP inhibit proliferation of patched mutant tumor cells

To examine the effects of targeting CD15+ TPCs using an immunotoxin approach, we treated patched mutant tumor cells with increasing concentrations of IgM-SAP and CD15-SAP. As shown in Figure 3.8 A, treatment of patched mutant tumor with CD15-SAP caused dramatic inhibition of proliferation. However, treatment with IgM-SAP also inhibited proliferation to the same extent, suggesting that the toxic effect of the CD15-SAP antibody was not mediated by specific binding or internalization of the antibody. The effects of these toxins were also assessed in additional patched mutant tumors (ranging from 18% to 60% CD15+), and substantial inhibition of proliferation upon treatment with IgM-SAP (with little to no additional effect of CD15-SAP) was
consistently observed (data not shown). One potential explanation for the lack of specificity of the CD15-SAP toxin could be that internalization of the toxin was not mediated by specific binding and internalization of the antibody into the cells. However, treatment with non-conjugated Saporin did not affect proliferation of patched mutant tumor cells (data not shown), suggesting that the toxic effects of IgM-SAP were indeed mediated by non-specific binding. Furthermore, pre-incubation of the cells with unconjugated IgM prior to treatment with IgM-SAP or CD15-SAP could eliminate the toxicity of the control IgM-SAP and led to significant specific inhibition of proliferation with CD15-SAP (Figure 3.8 B). While these data suggest that strategies can likely be developed to reduce the non-specific toxicity of the control immunotoxin in in vitro contexts, interpretation of in vivo results may prove challenging and not allow assessment of the effects of specifically eliminating TPCs from tumors. Therefore, alternative strategies for targeting CD15+ TPCs should be developed.
Figure 3.8: *patched* mutant tumor cells are sensitive to control IgM-SAP.

Figure 3.8: (A) *patched* mutant tumor cells were cultured in the presence of increasing concentrations of IgM-SAP or anti-CD15-SAP for 48 hours, then pulsed with \(^3\)H-Td and cultured for an additional 16 hours. (B) *patched* mutant tumor cells were pre-incubated with unconjugated IgM for 1 hour, then cultured in the presence of increasing concentrations of IgM-SAP or anti-CD15-SAP for 48 hours, pulsed with \(^3\)H-Td, and cultured for an additional 16 hours. Data represent average \(^3\)H-Td incorporation from triplicate wells for each condition.
3.4 Discussion

The identification of CD15 as a marker for tumor-propagating cells in *patched* mutant tumors has enabled the study of these cells, but the identity and function of the molecule carrying this epitope and strategies for targeting these cells have remained unknown. Here, we employed an immunoprecipitation approach to identify potential CD15 carrier proteins in *patched* mutant tumors. In addition, we utilized CD15 immunotoxins in an effort to specifically target these cells and assess their contribution to continued tumor growth. While these strategies were ultimately unsuccessful, they represent progress in our efforts to better understand these cells and suggest potential alternative strategies for achieving these goals.

To identify CD15 carrier proteins in *patched* mutant tumor cells, we performed immunoprecipitation using the same CD15 antibody (clone MMA) that was used to isolate CD15+ cells in the functional tumor propagation assay. Unfortunately, we were unable to identify any specific CD15-associated molecules. While immunoprecipitation approaches have been shown to successfully identify CD15-associated proteins in other contexts, the identity of the carrier appears to be dependent upon the antibody clone, even within the same tissue. For example, different CD15 antibody clones have identified the following molecules in neural stem cells: clone 5750 has identified Phosphacan, Tenascin-C, and L1-CAM; clone AK97 has identified β1-integrin, LAMP-1, and glycolipids; clone FORSE-1 has identified Phosphacan and neutral glycolipids; and

The different CD15 antibody clones have also been demonstrated to differentially recognize the fucose-galactose-acetylglucosamine epitope within different positions on the carrier molecule. For example, clone 5750 recognizes the epitope only within an internal position on the carbohydrate chain, clone SSEA-1 recognizes the carbohydrate only on long poly-N-acetyl lactosamine backbones, and clone 487 recognizes the epitope only on short carbohydrate backbones (Hennen et al. 2011) (Solter and Knowles 1978) (Fukui et al. 2002). The position of the epitope that is recognized by clone MMA, which was used in our studies, has not yet been characterized. However, the knowledge that differences exist in the ability of different antibodies to recognize the same epitope may provide one explanation for our inability to immunoprecipitate the CD15 carrier protein using the MMA antibody clone, since the affinities of the different antibody clones for a particular protein may also differ depending on the position of the epitope within the carbohydrate chain attached to the protein/lipid. It is possible that the affinity of the MMA clone for the CD15 carrier protein in patched mutant tumor cells is too low to immunoprecipitate a sufficient quantity of the protein for detection. To overcome this challenge, we could utilize another CD15 antibody clone for the immunoprecipitation experiments; however, to ensure that any proteins that are identified by a different clone
represent the CD15-associated molecules specifically on the tumor-propagating cells, we would also need to assess the tumor propagation capacity after isolating CD15+ and CD15- cells with the alternative antibody clone to confirm that the alternative antibody recognizes the same population of cells.

Given the identification of CD15-associated proteins in neural stem cells using other antibody clones, a candidate approach could also be used to determine whether the previously identified proteins contain the CD15 modification in patched mutant tumor cells. Although we did not exhaustively test candidate proteins, immunoprecipitation with antibodies against β1-integrin or phosphacan and analysis by CD15 western blot (or vice versa) failed to detect these as CD15-associated proteins in patched mutant tumor cells (data not shown). These data suggest that the epitope recognized by clone MMA that isolates the tumor-propagating cells in patched mutant tumors is not likely associated with expression of these proteins. Further analysis would be required to determine whether the CD15 epitope is present on the other previously described CD15 carrier proteins. However, even if this approach successfully identified the presence of the epitope on one of the candidate proteins, it would remain possible that additional unidentified proteins contain the CD15 modification and contribute to the tumor-propagating capacity of CD15+ cells.

Another possible explanation for our inability to identify a CD15-associated protein in patched mutant tumor cells may be that the CD15 epitope recognized by clone
MMA is predominantly expressed on glycolipids in these cells. While we did not pursue this avenue, immunoprecipitation followed by thin layer chromatography could be used to identify CD15-associated lipids. CD15-associated glycolipids, Galβ1-4(Fuca1-3)GlcNAcβ1-3Galβ1-4GlcNacβ1-3Galβ1-4Glcβ1-1’Cer and Galβ1-4(Fuca1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1’Cer, have previously been identified in the embryonic brain (Ngamukote et al. 2007) (Yanagisawa et al. 2005). Our data demonstrating loss of epitope recognition (as measured by flow cytometry) upon treatment of patched mutant tumor cells with methanol suggests that some proportion of the epitope is indeed expressed on lipids in these cells.

The presence of the epitope on a protein vs. lipid carrier may have implications for understanding the potential role for the carbohydrate in tumorigensis. While proteins are often thought to be responsible for signaling and adhesion, lipids are also capable of performing these functions, and the presence of the CD15 epitope on a lipid could be as significant (but perhaps more challenging to study) as its presence on a protein. Because we believe that CD15 is present on both proteins and lipids in patched mutant tumor cells, further studies to identify the specific carriers may enable an assessment of the relative contributions of the CD15-associated protein vs. lipid to tumorigensis.

While identification of the CD15 carrier molecule could ultimately suggest potential approaches for blocking the growth of CD15-expressing TPCs (such as through
small molecule or antibody-mediated inhibition of the CD15-associated protein), an alternative strategy for targeting is through the use of an immunotoxin. Targeted immunotoxins, delivered by local intratumoral administration by convection-enhanced delivery, are currently under clinical investigation for the treatment of malignant brain tumors (Candolfi et al. 2011) (Castro et al. 2011). One advantage to using an immunotoxin approach to target CD15+ cells is that knowledge of the CD15 carrier molecule is not required, because binding of the toxin to the target cells is mediated by the antibody-epitope recognition. If this approach enabled successful specific killing of CD15+ cells, but not CD15- cells, we could then assess the contribution of CD15+ cells to continued tumor growth within endogenous tumors and determine whether targeting of the tumor-propagating cells truly represents a strategy to more effectively eliminate tumors and prevent tumor recurrence.

Our observations indicated that saporin-conjugated CD15 antibody (clone MMA) can indeed inhibit proliferation and cause cell death of CD15-expressing cells in the F9 cell line, while the control IgM-SAP had no effect. However, unlike F9 cells, death of patched mutant tumor cells was observed upon treatment with saporin-conjugated control IgM, preventing an assessment of the effects of specific elimination of the CD15-expressing tumor-propagating cells. While the basis for the non-specific toxicity in patched tumor cells compared to F9 cells remains unknown, one possible explanation could be differences in the ability of IgM to bind to the cell surface. An Fc receptor for
IgM, FcmuR, which causes endocytosis of IgM antibodies upon binding, has been identified in other tissues and has been shown to be expressed in neural cell types (Nakahara et al. 2003) (Cho et al. 2010a). It is possible that patched mutant tumor cells express this receptor, while the F9 cell line does not. To test this hypothesis, we could examine the expression of FcmuR by flow cytometry or Western blot in patched mutant tumors and F9 cells. However, regardless of the mechanism of IgM internalization, the toxicity of the control IgM-SAP prevents assessment of the effects of selective killing of CD15+ cells. One approach to overcome this challenge would be to generate an immunotoxin based on an IgG antibody (or other antibody subclass) against CD15. Unfortunately, non-IgM antibodies against this epitope have not yet been developed and may be difficult to generate due to the widespread expression of the CD15 carbohydrate throughout many organisms.

Another immunotoxin-based approach for selectively targeting CD15+ cells could be to generate toxin-conjugated antibodies against other proteins that are expressed exclusively on the surface of CD15+ cells, but not on CD15- cells. Our lab has previously performed microarray analysis to identify differentially expressed genes in CD15+ and CD15- cells. The data from this analysis suggest that CD15+ cells contain elevated expression of multiple cell surface proteins, including VCAM-1 (Vascular cell adhesion molecule 1), CH1 (Neural cell adhesion molecule L1-like protein), CD36, Ptprz1 (phosphacan), and Itgb5 (integrin beta 5), among others (Read et al. 2009). While
further studies would be required to confirm the specificity of expression of these proteins in only CD15+ cells, differential expression could enable the use of non-IgM toxin-conjugated antibodies against these proteins as an alternative approach to specifically eliminate these cells.

Unfortunately, the strategies described above were unsuccessful in identifying the CD15-associated carrier molecules and in specifically targeting CD15+ cells in *patched* mutant tumors. However, other approaches to understand and target these tumor-propagating cells remain to be explored, including those that are not based upon the CD15 epitope itself. One such approach could utilize the microarray analysis of CD15+ and CD15- cells to identify pathways or properties that are particularly active in the CD15+ population. Chapter 4 will describe our efforts in pursuing this strategy to target CD15+ tumor-propagating cells.
4. Targeting Sonic Hedgehog-associated medulloblastoma through inhibition of Aurora and Polo-Like Kinases

4.1 Introduction

Medulloblastoma (MB) is the most common malignant pediatric brain tumor, with the majority of cases occurring in children under the age of 15 (Merchant et al. 2010). MB patients are commonly treated with surgery, radiation, and chemotherapy, but survivors suffer severe side effects, including cognitive and developmental deficits and an increased risk of secondary tumors later in life (Pollack 2011) (Crawford et al. 2007). Therefore, alternative approaches to treatment of MB are essential.

Recent genomic analyses have identified four major subtypes of MB that differ from one another in terms of gene expression, DNA copy number and mutation, epidemiology and prognosis. Although the genetic drivers of these subtypes are not fully understood, one group of tumors – representing ~25% of MB cases – is characterized by activation of the SHH signaling pathway. In some cases, this activation can be attributed to mutation or amplification of known pathway components, including the membrane proteins Patched (PTCH1) and Smoothened (SMO), the cytoplasmic regulator Suppressor of Fused (SUFU), and the transcription factors GLI1 and GLI2; however, in many cases, the basis for SHH pathway activation remains unclear (Taylor et al. 2012).
Patients with SHH-associated tumors have a variable prognosis, with a subset faring poorly despite aggressive therapy (Taylor et al. 2012). The development of small-molecule inhibitors of the SHH pathway has offered some hope for these patients, and several SHH antagonists are currently in clinical trials for MB (Metcalf and de Sauvage 2011) (Rudin et al. 2009). However, most of these compounds act on SMO and are thus unlikely to be effective against tumors driven by mutations in downstream components. Moreover, recent studies in both patients and animals have shown that PTCH- and SMO-driven tumors that initially respond to SHH antagonists quickly develop resistance (Rudin et al. 2009) (Yauch et al. 2009) (Buonamici et al. 2010). Thus, both SHH- and non-SHH-associated MBs will require more innovative approaches to therapy.

As discussed in Chapter 3, one approach to improving treatment of MB may involve targeting tumor-propagating cells (TPCs). We recently identified a population of TPCs in patched heterozygous mice, a widely studied mouse model of SHH-associated MB (Read et al. 2009). These cells can be identified based on their expression of the cell surface carbohydrate antigen CD15. Because CD15+ cells are critical for tumor propagation, we hypothesized that further understanding the properties of these cells might enable us to identify vulnerabilities that could be targeted by therapeutic intervention. Here, we report that CD15+ cells from patched mutant tumors display elevated expression of genes encoding regulators of G2 and M phases of the cell cycle.
and a corresponding over-representation of cells in G2/M phase. Furthermore, inhibition of Aurora kinases (Aurk) or Polo-like kinases (Plk), important G2/M regulators, inhibits proliferation in vitro and blocks tumor growth in vivo. Therefore, targeting TPCs through inhibition of G2/M regulators may represent a novel approach for improving treatment of patients with this disease.

4.2 Materials and Methods

4.2.1 Mice

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committees of Duke University and the Sanford-Burnham Medical Research Institute. Germline patched heterozygous mutant mice (Goodrich et al. 1996) were maintained by breeding with 129X1/SvJ or C57BL/6 mice from The Jackson Laboratory (Bar Harbor, ME). Conditional Math1-CreER; Ptcflx/flx mice (18) were treated with 0.8 mg of tamoxifen (T5648, Sigma, St. Louis, MI) in 40 μl of corn oil at post-natal day 4 to generate tumors 10-16 weeks later (Yang et al. 2008b). CD-1 Nu/Nu mice were obtained from Charles River Laboratories (Wilmington, MA), and NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ (NOD scid gamma; NSG) mice were obtained from The Jackson Laboratory.

4.2.2 Human tumor isolation and propagation

Human MB tissue for patient-derived xenografts was obtained from surgical resection of tumors at Duke University Medical Center (Durham, NC) or Rady
Children's Hospital (San Diego, CA). All procedures using human tissue were approved by the Institutional Review Boards of the respective institutions. Upon retrieval, the tissue was mechanically dissociated into a single-cell suspension, then immediately injected into the cerebella of NSG mice. When the mice became symptomatic, the tumors were again dissociated into single-cell suspensions and then re-transplanted back into the cerebella of naïve hosts to establish a propagated line for each patient-derived xenograft.

4.2.3 Chemicals

The Aurk inhibitors VX-680, PHA-739358, SNS-314, CYC116, AT9283, MLN8237, PHA-680602, CCT129202, ENMD-2076, and AZD1152-HQPA, the Plk inhibitors BI-2536, BI-6727, GSK461364, and ON-01910, and the chemotherapeutic agents Vincristine, Cisplatin, and Cyclophosphamide were obtained from Selleck Chemicals (Houston, TX). The SHH antagonist NVP-LDE225 was kindly provided by Novartis (Boston, MA).

4.2.4 Tumor cell isolation and culture

Tumors were obtained from germline patched heterozygous or conditional Math1-CreER; Ptc\textsuperscript{floxed/floxed} mice, and each experiment was performed multiple times using cells isolated from each strain. The complete tumor dissociation procedure has previously been described (Yang et al. 2008b) (Read et al. 2009). Briefly, tumors were digested in a papain solution to obtain a single-cell suspension, then centrifuged through a 35%-65% Percoll gradient. Cells from the 35%-65% interface were suspended
in Dulbecco’s Phosphate-Buffered Saline (DPBS) plus 5% Fetal Bovine Serum (FBS) for cell sorting or in NB-NS21 (Neurobasal with 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin/streptomycin, and NS-21 supplement (Chen et al. 2008)) plus 1% FBS (Invitrogen) for culture. The cells were plated on growth factor-reduced matrigel-(BD Biosciences, La Jolla, CA) coated plates.

4.2.5 Cell sorting

To obtain CD15+ and CD15- cell populations, cells were stained with control mouse IgM or anti-CD15 (clone MMA, BD Biosciences) antibodies, followed by anti-mouse IgM-phycoerythrin (PE) (Jackson ImmunoResearch, West Grove, PA). The cells were then sorted on a FACSVantage or FACSVantage SE DiVa flow cytometer (BD Biosciences). After sorting, the cells were pelleted and resuspended in NB-NS21 culture media or frozen until use for expression analysis.

4.2.6 Real-time PCR

Real-time PCR was performed to examine the mRNA expression levels of *AurkA*, *AurkB*, and *Plk1* in the CD15+ and CD15- populations. mRNA was prepared using an RNaseasy kit (QIAGEN Inc., Valencia, CA), and real-time PCR was performed using the QuantiTect SYBR Green RT-PCR kit (QIAGEN). Each reaction consisted of 10 ng of the appropriate RNA, 12.5 μl of 2X QuantiTect SYBR Green RT-PCR Master Mix, 1.25 μl of a 10 μM stock of the appropriate forward and reverse primers, 0.25 μl of QuantiTect RT mix, and RNase-free water in a total volume of 25 μl. The following primer sequences
were used: Aurora kinase A, F: GTTCCCTTCGGTCCGAAA, R: AATCATTTCCGGAGGCTG; Aurora kinase B, F: TCAGAAGGAGAAGCCTACCC, R: GACTCTCTGGGACACGTGTT; Polo-like kinase 1, F: ACGTCGTAGGCTTCCATGAC, R: CTGTTCAGGAGAGGTTGC; and Actin, F: TATTGGCAACGAGCGGTCC, R: GGCATAGAGGTCTTTACGGATGTC. Duplicate reactions were prepared without the QuantiTect RT mix to confirm the absence of genomic DNA contamination. The following reaction conditions were run on a Bio-Rad C1000 Thermal Cycler and CFX96 Real-time System (Bio-Rad Laboratories, Hercules, CA): reverse transcription at 50°C for 30 minutes, HotStarTaq DNA Polymerase activation at 95°C for 15 minutes, followed by 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Each reaction was analyzed in triplicate using the delta Ct method to determine the level of expression of each gene in the CD15+ population relative to the CD15- population in each tumor.

4.2.7 BrdU and cell cycle analysis

To monitor cell cycle kinetics, tumor cells were first sorted into CD15+ and CD15- populations as described above. After sorting, 2 million cells per well were plated into 24-well plates in NB-NS21 culture media. The cells were pulsed with BrdU for 30 minutes, then washed with media to remove any remaining BrdU. Cells were collected immediately after the pulse (“30 minutes”), or 6, 12, or 24 hours later, then fixed and stained using the FITC BrdU Flow Kit (BD Biosciences) and 7-Aminoactinomycin (7-
AAD) according to the manufacturer’s instructions. For cell cycle analysis of cells that were not labeled with BrdU, the same kit was used for fixation, permeabilization, and 7-AAD staining, but the anti-BrdU staining step was eliminated. The analysis was performed using a FACScan or FACSCanto flow cytometer (BD Biosciences) and FlowJo v.7.6.4 software (Tree Star, Inc., Ashland, OR).

4.2.8 Western blotting

To assess the levels of Histone H3 phosphorylation following treatment with inhibitors, cells were cultured in 24-well plates at a density of 2.5 million cells per well in the presence of the indicated concentrations of DMSO, VX-680, or BI-2536. Cells were then lysed in RIPA buffer (Millipore, Billerica, MA) containing 1 mM sodium orthovanadate, 2 mM sodium fluoride (both from Sigma, St. Louis, MO), and Complete, Mini, EDTA-free protease inhibitor tablets (Roche Applied Science, Indianapolis, IN). Proteins (30 μg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Invitrogen), which were then probed with antibodies against phospho-Histone H3 (Ser 10) (Millipore), total Histone H3 (Cell Signaling Technology, Danvers, MA), Actin (Santa Cruz Biotechnology, Santa Cruz, CA), or GAPDH (Cell Signaling Technology), followed by goat anti-rabbit antibodies conjugated to IRdye 680 (Rockland, Gilbertsville, PA). Proteins were detected using the Odyssey imaging system (LI-COR, Lincoln, NE).
4.2.9 Proliferation assays

To examine the effects of inhibitors on proliferation, tumor cells from patched mutant mice or human patient-derived xenografts were isolated as described above and plated in 96-well plates at a density of 0.2 million cells per well. Cells were cultured in the presence of the indicated concentrations of inhibitors for 48 hours in triplicate wells, then pulsed with [methyl-3H]thymidine (Amersham/GE Healthcare, Piscataway, NJ) and cultured for an additional 16–18 hours. Cells were harvested onto filters using a Mach IIIM Manual Harvester 96 (Tomtec, Hamden, CT), and incorporated radioactivity was quantified by liquid scintillation spectrophotometry on a Wallac MicroBeta scintillation counter (PerkinElmer, Waltham, MA).

4.2.10 In vivo drug administration

To assess the effects of Aurk or Plk inhibition on tumor growth, 8 million cells from patched mutant mice were suspended in 50% NB-NS21/50% growth-factor reduced matrigel and subcutaneously injected in a total volume of 100 μl into the flanks of CD-1 Nu/Nu mice. Tumors were measured using calipers, and tumor volumes were calculated using the formula volume = 0.52 x length x width² (Jensen et al. 2008). Drug treatment was initiated when tumors reached a volume of approximately 150 mm³. Animals were treated with 50 mg/kg BI-2536 (suspended in 0.1 N HCl, then diluted in saline) twice weekly via tail vein. Animals treated with PHA-739358 (30 mg/kg in 5% dextrose) were injected intraperitoneally twice daily. The animals were sacrificed when
the largest tumor volume in the cohort exceeded 2 cm³. After sacrifice, tumors were collected, weighed and photographed.

4.3 Results

4.3.1 CD15+ cells display elevated expression of G2/M regulators

To gain insight into the mechanisms underlying tumor propagation by CD15+ cells, we previously compared their gene expression profiles to those of CD15- cells from the same tumors (Read et al. 2009). Our analysis revealed that CD15+ cells express elevated levels of cell cycle regulators, and in particular, regulators of G2/M. To validate these data, we analyzed expression of several of these regulators by real-time RT-PCR. As shown in Figure 4.1A-C, expression of Aurora kinase A (AurkA), Aurora kinase B (AurkB), and Polo-like kinase 1 (Plk1) was significantly higher in the CD15+ population compared to the CD15- population in each tumor examined (n=3). These results suggest that CD15+ and CD15- cells can be distinguished based on their expression of G2/M regulators.
4.3.2 CD15+ cells are enriched in G2/M

The differential expression of G2/M regulators in CD15+ and CD15- cells suggested that these populations might differ in terms of cell cycle distribution. To examine this, we performed cell cycle analysis on freshly isolated CD15+ and CD15- cells. Analysis of multiple tumors (n=6) indicated that compared to the CD15- population, the CD15+ population contains a significantly higher proportion of cells in G2/M phase.
G2/M phase; ~20% of CD15+ cells reside in G2/M, compared to ~5% of CD15- cells (Figure 4.1D, E). A similar, but less pronounced, enrichment was seen in the proportion of CD15+ cells in S phase (data not shown). These data suggest that the elevated expression of G2/M regulators correlates with an increased proportion of CD15+ cells in G2/M phase.

**4.3.3 CD15+ cells progress more rapidly through the cell cycle than CD15- cells**

The increased proportion of CD15+ cells in G2/M phase could be explained by differences in cell cycle kinetics between the CD15+ and CD15- populations. To address this possibility, we pulse-labeled cells with BrdU and followed their progression through the cell cycle. CD15+ and CD15- cells from patched mutant tumors were cultured in the presence of BrdU for 30 minutes, and then washed and collected immediately for cell cycle analysis or cultured for an additional 6, 12, or 24 hours. As shown in Figure 4.2A and B, BrdU was incorporated into both CD15+ and CD15- cells; approximately 27% of the CD15+ cells incorporated the BrdU label, while only 7% of the CD15- cells were labeled. These data suggest that the over-representation of CD15+ cells in G2/M phase may result, in part, from an increased percentage of cells transiting through the cycle.
Figure 4.2: CD15+ cells progress more rapidly through the cell cycle than CD15- cells.

Figure 4.2: CD15+ and CD15- cells from germline patched mutant tumors were pulsed with BrdU for 30 minutes, washed, and then analyzed immediately (30 min) or cultured for an additional 6, 12 or 24 hours. (A, B) Flow cytometric analysis of BrdU (Y-axis) and DNA content (X-axis) of CD15- (A) and CD15+ (B) cells at the indicated time points. (C) Ratio of [percentage of cells in G2/M]:[percentage of cells in S phase] among the BrdU+ population at each time point. The percentage of CD15+ cells in G2/M phase increases much more rapidly than the percentage of CD15- cells in G2/M between 30 minutes and 12 hours, and CD15+ cells exit G2/M phase (indicated by drop in the G2/M to S ratio) sooner than CD15- cells.
To assess the kinetics with which each population proceeds through S phase and into G2/M, we examined the ratio of BrdU+ cells with 4N DNA (G2/M phase) to those with DNA content > 2N and < 4N (S phase) at each time point (Figure 4.2 C). Thirty minutes after the BrdU pulse, the CD15+ and CD15- populations included similar proportions of cells in S and G2/M (G2/M:S ratios = 1.05 and 1.07, respectively). However, as early as 6 hours after the BrdU pulse, CD15+ and CD15- cells began to exhibit differences in cell cycle distribution. In the CD15- population, the G2/M:S ratio increased slowly, to 1.24 at 6 hours, 1.45 at 12 hours, and 1.89 at 24 hours. In contrast, this ratio increased much more rapidly in the CD15+ population, reaching 1.92 at 6 hours and 2.42 at 12 hours. At 24 hours, the G2/M:S ratio in the CD15+ population dropped sharply, to 1.3, as many CD15+ cells exited G2/M and re-entered G1. Thus, CD15- cells require 24 hours to accumulate in G2/M phase at levels similar to those reached by the CD15+ cells within 6 hours. Based on these results, we conclude that CD15+ cells move through the cell cycle more rapidly than CD15- cells and that this rapid progression may also contribute to the over-representation of CD15+ cells in G2/M phase.

4.3.4 Targeting G2/M regulators blocks progression through the cell cycle and inhibits proliferation

The increased proportion of CD15+ cells in G2/M phase led us to hypothesize that *patched* mutant tumors might be sensitive to inhibitors of regulators of G2/M progression. To address this possibility, we treated tumor cells with the Aurk inhibitor
VX-680 (Tozasertib) and the Plk inhibitor BI-2536, and examined phosphorylation of Histone H3 on Serine 10. This residue is a direct target of AurkB (Hsu et al. 2000) (Harrington et al. 2004), and as shown in Figure 4.3 A, the Auk inhibitor VX-680 potently blocked its phosphorylation. Paradoxically, inhibitors of Plk1 have been reported to promote increased phosphorylation of Histone 3 on Serine 10 (Steegmaier et al. 2007) (Fink et al. 2007); consistent with this, we observed increased levels of phospho-H3Ser10 upon treatment with BI-2536 (Figure 4.3 B). These data suggest that inhibitors of Aurora and Polo-like kinases are active in patched mutant tumor cells.
Figure 4.3: Aurr and Plk inhibitors block proliferation and cell cycle progression.

Figure 4.3: Cells from conditional patched mutant tumors were cultured in the presence of the indicated concentrations of VX-680, BI-2536, or the corresponding percentages of DMSO. (A, B) Western blot analysis of the levels of phospho-Histone H3 (Ser10) after 6 hours of treatment with
VX-680 (A) or BI-2536 (B). Note that Aurk inhibition decreases phosphorylation whereas Plk inhibition increases it. (C) Effects of Aurk and Plk inhibitors on proliferation. Cells were cultured with DMSO, VX-680 or BI-2536, pulsed with tritiated thymidine (3H-Td) at 48 hours, and harvested at 66 hours for analysis of 3H-Td incorporation. Data represent means of triplicate wells ± SEM. Treatment with 20 nM, 100 nM and 500 nM BI-2536 or VX-680 significantly inhibited 3H incorporation compared to corresponding DMSO controls (p < 0.01, based on paired 2-tailed t-test). (D-G) Flow cytometric analysis of DNA content, and graphical representation of percentages of cells in each cell cycle phase, after treatment with DMSO (D), 100 nM LDE-225 (E), 100 nM VX-680 (F), or 100 nM BI-2536 (G).

To determine the effects of Aurk and Plk inhibitors on proliferation, we performed 3H-thymidine incorporation assays. As shown in Figure 4.3 C, treatment with 100 nM or 500 nM VX-680 or BI-2536 caused nearly complete inhibition of proliferation. To define the IC₅₀ values for VX-680 and BI-2536, we treated cells with increasing concentrations of these compounds (0.15 nM - 1.5 μM) and measured 3H-thymidine incorporation. As shown in Figure 4.4 A and B, the IC₅₀ values for VX-680 and BI-2536 were 23 nM and 4.5 nM, respectively. These values are consistent with previously reported IC₅₀ values for these drugs in other types of tumor cells (Harrington et al. 2004) (Steegmaier et al. 2007). To further validate the anti-proliferative effects of Aurk and Plk inhibition, we assessed the sensitivity of patched mutant tumor cells to additional Aurk or Plk inhibitors. As shown in Figure 4.4 C and D, multiple Aurk and Plk inhibitors displayed potent anti-proliferative effects. These data confirm that patched mutant tumor cells are vulnerable to small molecule-mediated inhibition of Aurk or Plk activity.
Figure 4.4: Avid and Plk inhibitors block proliferation of patched mutant

Figure 4.4: (A,B) Cells from conditional patched mutant tumors were cultured in the presence of the indicated concentrations of VX-680 (A) or BI-2356 (B), pulsed with ³H-Td at 48 hours, and harvested for analysis of ³H-Td incorporation at 66 hours. IC50 values were calculated using the log(inhibitor) vs. response equation \( Y=\text{Bottom}+\frac{\text{Top}-\text{Bottom}}{1+10^{(X-\log\text{IC50})}} \) in GraphPad Prism software. (C, D) Germline patched mutant tumor cells were treated with the SHH antagonist LDE-225 or the indicated Avid inhibitors (C) or Plk inhibitors (D), and ³H-Td incorporation was assessed as described above. Data represent means of triplicate samples ± SEM. Avid inhibitors significantly reduced ³H-Td incorporation \((p < 0.05\) based on paired 2-tailed t-tests), with the exceptions of PHA-739358 (100 nM), CYC116 (100 nM), PHA-680602 (100 nM), CCT129202 (500 nM and 100 nM), and ENMD-2076 (500 nM and 100 nM). All Plk inhibitors significantly reduced ³H-Td incorporation \((p < 0.005\).
To assess the effects of these inhibitors on cell cycle progression, we treated *patched* mutant tumor cells with VX-680 or BI-2536 for 24 or 48 hours. For comparison, we also treated cells with the SHH antagonist NVP-LDE225 (LDE-225), which is currently in clinical trials for the treatment of SHH-associated MB (Miller-Moslin et al. 2009) (Pan 2010). As shown in Figure 4.3 D and E, exposure to LDE-225 caused a progressive decrease in the number of cells in G2/M and a concomitant accumulation of cells in G1. In contrast, both VX-680 and BI-2536 markedly increased the number of cells in G2/M, while decreasing the G1 population (Figure 4.3 D, F, and G). Treatment with each of these inhibitors also caused an increase in the proportion of cells with < 2N DNA, most likely representing apoptotic cells. These data suggest that *patched* mutant tumor cells are sensitive to Aurk or Plk inhibition and that the effects of these inhibitors on the cell cycle are distinct from those induced by inhibitors of the SHH pathway.

### 4.3.5 Plk inhibition cooperates with SHH antagonist and conventional chemotherapy

Given the distinct effects of LDE-225 and VX-680 or BI-2536 on cell cycle progression, we hypothesized that these drugs might exert complementary or cooperative effects. To address this possibility, we treated tumor cells with increasing concentrations (0.15 - 1500 nM) of LDE-225 either with or without 10 nM BI-2536, a concentration of BI-2536 that caused minimal inhibition of proliferation on its own. As shown in Figure 4.5 A, treatment with LDE-225 alone inhibited proliferation at concentrations above 15 nM, with an IC50 of ~9 nM. However, concomitant treatment
with 10 nM BI-2536 enhanced the inhibition of proliferation at all concentrations of LDE-225 and caused the IC50 for LDE-225 to shift to ~2 nM, suggesting that BI-2536 cooperates with LDE-225 to inhibit proliferation. Similar cooperation was observed between VX-680 and LDE-225 (Figure 4.6 A).
Figure 4.5: Plk inhibitor cooperates with SHH antagonist and chemotherapeutic agents.

Figure 4.5: Conditional patched mutant tumor cells were treated with increasing concentrations of the SHH antagonist LDE-225 (A) or the chemotherapeutic agents Vincristine (B), Cisplatin (C), or Cyclophosphamide (D), alone or in combination with 10 nM BI-2536. Cells were cultured for 48 hours, pulsed with $^3$H-Td, and harvested for analysis of $^3$H-Td incorporation at 66 hours. Data represent means of triplicate samples ± SEM. IC50 values were calculated using the log(inhibitor) vs. response equation ($Y=$Bottom + (Top-Bottom)/(1+$10^8$($(X-Log(IC50))$)) in GraphPad Prism software.
Figure 4.6: Aurk inhibitor cooperates with SHH antagonist and chemotherapeutic agents.

Figure 4.6: Conditional patched mutant tumor cells were treated with increasing concentrations of LDE-225 (A), Vincristine (B), Cisplatin (C), or Cyclophosphamide (D) alone or in combination with 30 nM VX-680, pulsed with ³H-Td at 48 hours, and harvested for analysis of ³H-Td incorporation at 66 hours. IC50 values were calculated using the log(inhibitor) vs. response equation ($Y=Bottom + (Top-Bottom)/(1+10^{(X-\text{LogIC50})})$) in GraphPad Prism software.
To determine whether G2/M inhibitors can also cooperate with conventional chemotherapeutic agents, we treated patched mutant tumor cells with 10 nM BI-2536 in combination with Vincristine, Cisplatin, and Cyclophosphamide, chemotherapeutic agents that are currently being used to treat human MB (26). Addition of BI-2536 markedly increased the sensitivity of the tumor cells to Vincristine; while the IC50 value for Vincristine alone was approximately 5 nM, the IC50 value for Vincristine combined with BI-2536 was approximately 0.1 nM (Figure 4.5 B). Similar, but less dramatic, cooperation was observed with Cisplatin (IC50 = 44 nM for Cisplatin alone and 34 nM for Cisplatin + BI-2536) and with Cyclophosphamide (IC50 = 10 μM for Cyclophosphamide alone and 8 μM for Cyclophosphamide + BI-2536) (Figure 4.5 C and D). VX-680 also cooperated with Vincristine and Cyclophosphamide (Figure 4.6 B-D). These data suggest that the addition of BI-2536 or VX-680 can lower the concentrations of chemotherapy required for effective inhibition of tumor cell proliferation.

4.3.6 Inhibition of G2/M regulators blocks tumor growth in vivo

Given the strong anti-proliferative effects of the Aurk and Plk inhibitors in vitro, we next questioned whether inhibition of G2/M regulators could inhibit tumor growth in vivo. Because the Plk inhibitor BI-2536 has demonstrated promising results in clinical trials completed thus far (27-29), we prioritized this compound for our in vivo studies. patched mutant tumor cells were implanted subcutaneously into the flanks of Nu/Nu mice, and two weeks later, treatment was initiated with either vehicle or BI-2536 (50
mg/kg twice weekly via tail vein). As shown in Figure 4.7 A, BI-2536 markedly inhibited tumor growth, as measured by tumor volume over time. Upon harvesting the tumors (~2.5 weeks after starting treatment), substantial differences in tumor size and weight were observed (Figure 4.7 B and C). Overall, tumors from the BI-2536-treated mice were significantly smaller and weighed less than tumors from the vehicle-treated mice. In addition, tumors from the BI-2536-treated mice showed little evidence of angiogenesis, unlike the extensively angiogenic tumors from the vehicle-treated mice (Figure 4.7 B). The Aurk inhibitor PHA-739358 also blocked tumor growth and led to a reduction in tumor size and weight (Figure 4.8 A, B, and C). Collectively, these data suggest that inhibition of G2/M regulators can effectively block tumor progression in vivo.
Figure 4.7: Plk inhibitor blocks the growth of patched tumors in vivo.

Figure 4.7: Mice bearing subcutaneous allografts of conditional patched mutant tumor cells were treated twice weekly with vehicle (saline) or 50 mg/kg BI-2536. (A) Tumor volume (mm$^3$) was measured using calipers. Arrow indicates start of treatment, and each line represents an individual mouse. (B) Images of tumors. (C) Tumor weights. Each point represents a single tumor, and grey lines represent mean tumor weights, which were significantly different between vehicle and BI-2536 treated mice (p < 0.05, based on paired two-tailed t-test).
Figure 4.8: Mice bearing subcutaneous allografts of germline *patched* mutant tumor cells were treated twice daily with vehicle (5% Dextrose) or 30 mg/kg PHA-739358. (A) Tumor volume was measured using calipers. Arrows indicate start of treatment, and each line represents an individual mouse. (B) Images of tumors. (C) Tumor weights. Each point represents a single tumor, and grey lines represent the mean tumor weights, which were significantly different (p < 0.01, based on paired two-tailed t-test between the vehicle and PHA-739358 group).
4.3.7 Aurk and Plk inhibitors suppress growth of human SHH-associated MB

The studies above focused on tumors from patched mutant mice. To determine whether G2/M inhibitors might also be effective against human MB, we used cells from patient-derived MB xenografts. As shown in Figure 4.9 A, treatment of cells from a human SHH-associated MB (DMB-012) with BI-2536 caused a marked inhibition of proliferation, comparable to that seen with the SHH antagonist LDE-225. In addition, RCMB-018 cells, derived from a SHH-associated MB that is insensitive to LDE-225 (due to amplification of SHH pathway components downstream of SMO), were also inhibited by BI-2536 (Figure 4.9 B). These data suggest G2/M inhibitors might be useful for treating human SHH-associated tumors, including those that display resistance to SHH antagonists.
Figure 4.9: Aurk and Plk inhibitors suppress proliferation of human SHH-associated medulloblastoma.

Figure 4.9: (A, B) Cells from patient-derived xenografts of SHH-associated MB that are sensitive (DMB-012, panel A) or resistant (RCMB-018, panel B) to SHH antagonists were treated with DMSO (0.25%), LDE-225 (500 nM), or BI-2536 (500 nM). Cells were pulsed with ³H-Td after 48 hours and harvested for analysis of ³H-Td incorporation at 66 hours. In DMB-012, LDE-225 and BI-2536 significantly inhibited ³H incorporation compared to DMSO control (p < 0.01 based on paired two-tailed t-test). In RCMB-018, BI-2536 caused significant inhibition (p = 0.01), whereas LDE-225 did not (p = 0.89). (C) DMB-012 cells were cultured in the presence of LDE-225 (100 nM), PHA-739358 (100 nM) or the combination of LDE-225 + PHA-739358, and assayed for 3H-Td incorporation as described above. Data represent means of triplicate samples ± SEM. ³H-Td incorporation in the presence of LDE-225 + PHA-739358 was significantly lower than in the presence of LDE-225 alone (p = 0.05) or PHA-739358 alone (p = 0.004).
To address whether Aurk inhibition and SHH pathway antagonism cooperate in human MB, we treated DMB-012 cells with intermediate concentrations of the Aurk inhibitor PHA-739358 and the SHH antagonist LDE-225. As shown in Figure 4.9 C, treatment with 100 nM LDE-225 alone or 100 nM PHA-739358 alone caused inhibition of proliferation, as expected. However, treatment with 100 nM LDE-225 together with 100 nM PHA-739358 further inhibited proliferation beyond that of either compound alone. These data suggest that, similar to the results observed in cells from patched mutant tumors, inhibition of G2/M regulators can cooperate with SHH pathway antagonism to block the growth of human SHH-associated MB.

4.4 Discussion

Although treatment for MB has dramatically improved survival in recent years, patients often suffer severe side effects, and better treatment strategies are still required. Targeting MB TPCs represents one approach to improving treatment. Using the patched mutant mouse model of MB, we have demonstrated that CD15+ TPCs disproportionately reside in G2/M phase and that inhibition of Aurora kinases or Polo-like kinases can inhibit tumor growth in vitro and in vivo. In addition, we have shown that these inhibitors can block the growth of cells from patient-derived xenografts of human MB. Our data suggest that incorporating Aurk or Plk inhibitors into MB therapy could lead to improvements in treatment outcome.
Our previous studies indicated that CD15+ TPCs from *patched* mutant tumors display elevated expression of G2 and M phase cell cycle regulators (Read et al. 2009). Here, we confirmed increased expression of AurkA, AurkB, and Plk1. Each of these serine/threonine kinases plays a distinct role in G2/M phase progression (Lens et al. 2010). AurkA is involved in centrosome duplication, bipolar spindle assembly, and entry into mitosis, while AurkB functions in chromatin modification, microtubule-kinetochore attachment, spindle assembly checkpoint activation, and cytokinesis. Plk1 is involved in centrosome separation, spindle assembly and maturation, cytokinesis, and exit from mitosis. Overexpression of each of these kinases has been associated with poor prognosis in multiple tumor types, leading to the notion that elevated expression of these proteins might promote tumor growth (Neben et al. 2004) (Ali et al. 2012) (Lehman et al. 2012) (Liang et al. 2012) (Cheng et al. 2012; King et al. 2012). However, given the elevated expression of multiple G2/M regulators in CD15+ cells, we hypothesized that the expression profile reflected a general property of the CD15+ population, rather than a reliance on elevated expression of a single kinase to drive tumorigenicity. Cell cycle analysis of CD15+ and CD15- populations from multiple tumors demonstrated an increased percentage of the CD15+ population residing in G2/M phase compared to the CD15- population, suggesting that the elevated expression of G2/M regulators in the CD15+ population is likely a result, rather than a cause, of the increased percentage of cells residing in G2/M.
Multiple factors could potentially contribute to the accumulation of CD15+ cells in G2/M. One explanation is that a greater overall percentage of CD15+ cells transit through the cell cycle, while CD15- cells remain largely stationary in G0/G1 phase. This notion is supported by the observation that the CD15+ population also contains a greater fraction of cells in S phase than the CD15- population. Another possible explanation for the accumulation in G2/M could be that the CD15+ cells arrest in these phases of the cell cycle. To address this possibility, we performed BrdU labeling and cell cycle analysis to monitor the position of the BrdU-labeled cells in the cell cycle over time. We observed greater incorporation of the BrdU label in CD15+ cells, which again suggested that greater numbers of CD15+ cells transit through the cell cycle. However, examination of the BrdU-labeled cells over time indicated that CD15+ cells do not arrest in G2/M, but actually progress more rapidly through the cell cycle than CD15- cells. While these data do not exclude the possibility of transient checkpoint activation and/or cell cycle arrest, they indicate that cell cycle arrest is not a primary contributor to the over-representation of CD15+ cells in G2/M phase. Furthermore, the data suggest that both increased numbers of cycling cells and an increased pace of progression through the cell cycle contribute to the over-representation of CD15+ cells in G2/M phase.

We speculated that the increased residency of CD15+ cells in G2/M phase could represent a vulnerability of these cells that could be targeted through inhibition of G2/M regulators. Because small molecule inhibitors of the Aurora kinases and Polo-like
kinases have shown promising efficacy in Phase I and Phase II clinical trials for other tumor types, we selected the Aurk inhibitor VX-680 and Plk inhibitor BI-2536 for evaluation in our studies (Cheung et al. 2011) (Chopra et al. 2010) (Traynor et al. 2011) (Hofheinz et al. 2010) (Sebastian et al. 2010) (Mross et al. 2008). Our data demonstrate that patched mutant tumors are indeed sensitive to Aurk or Plk inhibition; both VX-680 and BI-2536 effectively blocked proliferation in vitro.

One principal aim of this study was to identify approaches that might enhance current MB therapy. SHH antagonists have recently been developed for treatment of human SHH-associated MB (Metcalfe and de Sauvage 2011). Both patients and mice who receive these antagonists initially respond to treatment, but they quickly develop resistance (Rudin et al. 2009) (Yauch et al. 2009) (Buonamici et al. 2010). Previous studies have shown that SHH signaling regulates the transition between G1 and S phases of the cell cycle (Adolphe et al. 2006) (Cayuso et al. 2006) (Kenney and Rowitch 2000) (Oliver et al. 2003); consistent with these observations, our data indicate that the SHH antagonist LDE-225 causes dramatic accumulation in G1 phase. In contrast, the Aurk and Plk inhibitors cause accumulation in G2/M phase. Given these distinct mechanisms of cell cycle inhibition, we speculated that blocking G2/M progression might represent an additional point of intervention to target the cells that escape sensitivity to SHH antagonists. Our data demonstrate that the combination of LDE-225 plus BI-2536 or VX-680 has a greater inhibitory effect than treatment with any compound alone. These data
suggest that clinical combination of SHH antagonists plus Aurk or Plk inhibitors might enhance the efficacy of therapy and prevent the acquired resistance to SHH antagonists.

Although the development of SHH antagonists has provided additional options for therapy, most MB patients are still treated with conventional chemotherapy. However, this treatment is extremely toxic and causes significant side effects (Pollack 2011). We questioned whether treatment with chemotherapy plus the Aurk or Plk inhibitors might enable a reduction in the dose of chemotherapy while maintaining the efficacy of treatment. Our data demonstrate that the combination of individual chemotherapy agents (Vincristine, Cisplatin, or Cyclophosphamide) plus BI-2536 or VX-680 is more effective than either the chemotherapy agent, BI-2536, or VX-680 alone. Previous studies have shown similar effects of combined treatment of MB cell lines with chemotherapy or radiation plus Aurk or Plk inhibitors (Harris et al. 2012) (El-Sheikh et al. 2010) (Muscal et al. 2012). Together, these studies suggest the potential utility of combination therapy, although the mechanism for cooperation may differ for each chemotherapy agent. Inhibitors that cause G2/M cell cycle arrest may cooperate with chemotherapy agents that exert their cytotoxic effects in G2/M (such as the microtubule polymerization inhibitor Vincristine). In contrast, cell cycle arrest in G2/M may not cooperate as strongly with chemotherapy agents that act in S phase, such as DNA alkylating agents (including Cyclophosphamide). The data presented here suggest that incorporating treatment with Aurk or Plk inhibitors into a chemotherapy regimen for
the treatment of MB might enable a reduction in the chemotherapy dose and improve quality of life.

The anti-proliferative effects of the Aurk and Plk inhibitors in vitro prompted us to examine the effects of the inhibitors in vivo. Our data indicate that treatment of mice harboring subcutaneous allografts of patched mutant tumors with BI-2536 blocks tumor growth in vivo. Interestingly, a decrease in tumor blood vessel formation was also observed in mice treated with BI-2536. While the observed inhibition of tumor growth in animals treated with the Plk inhibitor is consistent with the expected role of Plk1, further studies are required to examine the mechanism by which Plk inhibitors influence angiogenesis in these tumors. However, the significant inhibition of in vivo tumor growth upon treatment with BI-2536 validates the notion that targeting a vulnerability of the TPC population can block tumor growth and suggests that inhibition of Plk may represent a viable approach for MB treatment.

By examining the TPC population in the patched mutant mouse model of MB, we have identified an opportunity for therapeutic intervention. Our data indicate that cells from patient-derived xenografts of human SHH-associated MB are also sensitive to Aurk or Plk inhibition. Importantly, tumor cells that are insensitive to SHH antagonists maintain sensitivity to BI-2536, validating the notion that treatment with the Plk inhibitor may represent an approach to overcome therapeutic resistance to SHH antagonist therapy. In addition, similar to the patched mutant tumor cells, treatment of
human tumor cells with an Aurk inhibitor plus LDE-225 blocks proliferation more effectively than either compound alone. These data suggest that mouse and human SHH-associated MB are sensitive to Aurk or Plk inhibition, and strongly support the notion of incorporating Aurk or Plk inhibitors into therapeutic strategies for improving the outcome of patients with MB.
5. Conclusions and perspectives

Elucidation of the signals that regulate cerebellar development and tumor growth are necessary for a better understanding of neurogenesis and improved approaches for treating tumors. The studies described here have advanced our understanding of the signals that control normal migration in the developing cerebellum and have generated potential avenues for improving the treatment of medulloblastoma. The use of mouse models was critical for these studies, as these objectives cannot be fully addressed with other commonly used tools. Understanding the signals that are required for proper migration requires an assessment of perturbing these signals in their normal developmental environment, which cannot be fully accomplished using cell culture approaches. Similarly, given the relative inaccessibility of primary patient tumors and the limited capacity of tumor cell lines to fully recapitulate all aspects of tumor biology, the use of the patched mutant tumor model was critical to study and identify vulnerabilities of tumor-propagating cells derived from endogenous tumors. While further studies are required, the work described here will hopefully contribute to a better understanding of cerebellar development and to improving treatment for patients with medulloblastoma.
5.1 CXCR4 is required for appropriate migration in the cerebellum

Elucidation of the signals that govern cerebellar development is necessary to understand the deregulation that can occur during development and result in disease. As in all tissues, cerebellar development proceeds through tightly coordinated processes of proliferation, migration, and differentiation, and the appropriate regulation of these processes is critical for the formation of the mature, properly functioning cerebellar structure. In our studies, we examined the influence of CXCR4 on these processes in granule neuron progenitors (GNPs). As described in Chapter 2, we have shown that CXCR4 is required for the appropriate radial migration of GNPs during cerebellar development. By crossing Math1-Cre mice with CXCR4\textsuperscript{flox/flox} mice, we were able to assess the effects of CXCR4 deletion specifically in GNPs, which enabled the mice to survive past birth (unlike CXCR4\textsuperscript{−/−} or SDF-1\textsuperscript{−/−} mice) and allowed evaluation of the postnatal consequences of CXCR4 deletion.

Our analysis demonstrated that loss of CXCR4 causes premature migration of clusters of GNPs from the EGL into the IGL and white matter. While loss of CXCR4 was associated with decreases in Shh pathway target gene expression, CXCR4-deficient GNPs were able to proliferate normally both \textit{in vitro} and \textit{in vivo} and could ultimately differentiate into mature granule neurons, regardless of their location. Furthermore, CXCR4 deletion in GNPs did not affect tumor formation in \textit{patched} mutant mice, suggesting that CXCR4 is dispensable for tumorigenesis. Together, our data suggest
that CXCR4 is not required for proliferation or differentiation of GNPs but primarily contributes to cerebellar development by regulating GNP migration.

The observation that only a subset of cells display the premature migration phenotype is surprising, given that CXCR4 expression is lost in all GNPs in Math1-Cre; CXCR4\textsuperscript{floxflo} mice. Notably, the conventional CXCR4\textsuperscript{−/−} and SDF-1\textsuperscript{−/−} mice (in which CXCR4 expression is lost in all cells) also display this phenotype in a subset of cells. These data suggest that while CXCR4 is clearly one factor that contributes to migration, redundancy likely exists in the system, and other additional factors likely regulate this process in cells that are not affected by loss of CXCR4. Even within the subset of cells that are affected by loss of CXCR4, it is possible that additional factors can also contribute to radial migration, but are not sufficient to prevent the observed phenotype. How these redundant signals coordinate with each other to regulate migration and whether there are specific subsets of cells that are dependent upon individual different factors remains to be determined. While it is also possible that the cells that are affected by loss of CXCR4 represent a functional subset of GNPs with distinct fates, further studies would be required to examine this possibility.

Another surprising outcome from our studies was the observation that the ectopic cell clusters in Math1-Cre; CXCR4\textsuperscript{floxflo} mice can continue to proliferate normally outside of their normal proliferative niche in the EGL and can ultimately differentiate at the proper time in aberrant locations outside of the IGL. Studies of normal cerebellar
development have suggested that an inextricable linkage exists between GNP proliferation, migration, and differentiation, such that exit from the EGL is tightly associated with the cessation of proliferation and the initiation of differentiation (Choi et al. 2005; Roussel and Hatten 2011). Our results are among the first to demonstrate that continued proliferation is possible outside of the EGL, and that migration is not absolutely coupled with differentiation.

The sustained proliferation of CXCR4‐deficient GNPs outside of the EGL raises the question of how these aberrantly migrating GNPs are capable of continued proliferation and appropriate differentiation in the “wrong” microenvironments. One possibility is that the ectopic cells contain an intrinsic program or factors to support their own proliferation. For example, although Purkinje cells are believed to be the predominant source of the Shh mitogen, there have been reports that GNPs themselves can produce and secrete Shh (Dahmane and Ruiz i Altaba 1999; Wechsler-Reya and Scott 1999). This raises the possibility that because the ectopic cells migrate in clusters, they can respond to Shh secreted by their neighboring ectopic cells. Alternatively, the observation that occasional Purkinje cell bodies are associated with the ectopic clusters may suggest that the aberrantly migrating GNPs recruit or physically push the Purkinje cells, causing them to remain in proximity to the GNP clusters, thereby ensuring a continued supply of their normal source of Shh.
The differentiation of ectopic GNPs may also be similarly regulated by intrinsic or extrinsic factors. The ability of the ectopic GNPs to differentiate into mature granule neurons at the proper time in the wrong location may suggest that the cells contain an intrinsic “clock” that recognizes the number of divisions and regulates the switch from proliferation to differentiation. Alternatively, similar to the observed phenomenon of association with Purkinje cells, the ectopic clusters may recruit cells or factors that promote their differentiation. The ectopic cells may thereby create their own traveling microenvironmental niche to support their survival and differentiation. Further studies will be required to elucidate the mechanisms (whether intrinsic or extrinsic) by which the continued proliferation and appropriate differentiation of the ectopic GNPs is possible.

Signals that regulate migration can also contribute to tumor growth. Tumor initiation may require a specific microenvironment, and disruption of the signals that define the location of the initiating cells might affect tumor formation and/or maintenance. Tumors in the SHH-associated subset of medulloblastoma have been shown to be located on the surface of the cerebellum in both mice and humans, and cells that are capable of remaining in the EGL might have a growth advantage (Oliver et al. 2005; Gibson et al. 2010). Because the SDF-1/CXCR4 interaction can contribute to maintaining cells within this environment, loss of CXCR4 in GNPs might be expected to prevent the formation of SHH-associated tumors. However, our studies have indicated
that CXCR4-deficient GNPs are capable of tumor formation upon loss of patched, suggesting that CXCR4 is not required for tumorigenesis. This result may not be surprising, given that the majority of GNPs are unaffected by loss of CXCR4 and remain in the EGL for the proper amount of time. In addition, because the prematurely migrating cells that are affected by loss of CXCR4 are capable of continued proliferation in these animals, proximity to the surface of the cerebellum may not be necessary for tumor formation in this context.

Regardless of the requirement for CXCR4 in tumor initiation, the possibility remains that CXCR4-expressing established tumors may be dependent upon CXCR4 to mediate interaction with the meninges or blood vessels for their maintenance. Previous studies have indicated that mouse SHH-associated tumors are sensitive to small molecule-mediated inhibition of CXCR4 signaling (Sengupta et al. 2012). Although our observations are not consistent with these data, the discrepancy could be explained by compensation over the course of GNP and tumor development in our genetic system. Further studies to elucidate the mechanisms for this compensation in normal GNPs and tumors may enable the identification of additional therapeutic targets in SHH-associated tumors that do not display elevated expression of CXCR4.

Together, the observations that only a subset of cells are affected by loss of CXCR4 signaling and the ability of these cells to properly proliferate and differentiate suggest that perturbation of the overall system of cerebellar patterning and development
is very difficult. The complexity and redundancy in the factors and cells that regulate development is likely a result of evolutionary processes that ensure that these mechanisms lead to the formation of a properly functioning cerebellum. However, given the redundancy, a break-down in the system requires severe aberrations that can have devastating consequences, such as the development of cancer. A better understanding of the complexity and redundancy mechanisms during normal development will ultimately enable recognition of the deregulation that leads to cancer (or other cerebellar defects) and identification of approaches to restore normal homeostasis.

5.2 Identification of CD15-associated carrier molecules and approaches for targeting CD15+ tumor-propagating cells

While Chapter 2 focused on understanding the signals that regulate normal cerebellar development, the studies described in Chapters 3 and 4 concentrated on improving our understanding of tumor-propagating cells in established tumors and identifying approaches for targeting them. The identification of CD15 as a marker for tumor-propagating cells led to the following two important questions: (1) what enables these cells to propagate tumors; and (2) can targeting these cells block tumor growth? One strategy to address these questions is based on an understanding of the CD15 carrier molecules in patched mutant tumors. Chapter 3 discussed our efforts to identify the CD15-associated molecule using an immunoprecipitation approach, with the goal of performing mass spectrometry to identify the isolated protein. Unfortunately, although
this approach could successfully immunoprecipitate a high molecular weight protein in F9 cells (as previously reported), we were unable to detect any specific CD15-associated protein in patched mutant tumor cells. However, treatment of CD15-expressing patched mutant tumor cells with either enzymes to remove proteins or with methanol to remove lipids led to a decrease in CD15 antibody binding, suggesting that both proteins and lipids may act as CD15 carrier molecules in patched tumor cells.

Further studies will be required to identify the specific CD15 carrier molecules in patched mutant tumor cells (possibly using alternative CD15 antibodies, thin layer chromatography, or other approaches). Identification of the CD15-associated molecules will then enable future studies to determine the role of the molecule(s) in tumor formation, maintenance, and propagation. The approaches used for these studies will likely be dictated by the nature of the molecule. The identification of a CD15-associated protein would enable studies that employ shRNAs, blocking antibodies, small molecule inhibitors, or conditional mouse models to determine the function of the protein in tumor growth. If the CD15-associated protein is found to be required to mediate the tumor-propagation capacity of these cells, blocking antibodies or small molecule inhibitors could also be considered for development as therapeutic agents. In contrast, the identification of CD15-associated lipids might require different approaches to elucidate its function. These approaches may employ disruption of the enzymes that mediate glycolipid assembly or processing.
Identification of the CD15-associated molecule could provide insight into the mechanism by which CD15+ cells are capable of tumor propagation. Currently, the basis for why CD15+ cells have this capacity remains unknown, but one possible explanation could be that they have a unique ability to adhere or migrate to or establish the “proper” microenvironment to support continued growth. Another (non-exclusive) possibility could be that these cells have activation of specific (as yet unknown) signaling pathways that prevent cell cycle exit and/or differentiation. Why expression of the CD15 epitope is linked to this capacity also remains unknown, but identification of the carrier molecule and assessment of its function in patched mutant tumors may address whether this expression itself promotes tumor propagation or is a consequence of activation of other drivers of proliferation or tumorigenesis. Future studies that examine the mechanisms that regulate CD15 expression on the carrier molecule may enable a better understanding of why the epitope can serve as a marker of the tumor-propagating cells.

Although tumor-propagating cells are believed to be responsible for tumor recurrence after therapy based on their ability to propagating tumors in transplant-based systems, the role of TPCs in endogenous tumor growth has yet to be fully defined. Can targeting TPCs in a primary tumor (non-transplant) setting truly eliminate the capacity for tumor recurrence? To address this question, we developed an anti-CD15 immunotoxin in an attempt to specifically target TPCs in patched mutant tumor cells.
However, while our studies showed that the CD15-SAP immunotoxin can selectively kill the CD15-expressing F9 cell line (but not the CD15-negative Ba/F3 cell line), the toxicity of the control IgM-SAP in patched tumor cells was comparable to that of CD15-SAP, thwarting conclusive analysis of the effects of targeting the TPC population. Therefore, alternative approaches will likely be necessary to target this population.

The identification of the CD15 carrier molecule may enable several strategies for targeting these cells. For example, alternative non-IgM toxin-conjugated antibodies against the protein or lipid (rather than the CD15 epitope) or toxin-conjugated ligand (if the CD15-associated molecule acts as a receptor) could potentially be used to target these cells. Alternatively, if the carrier molecule is functionally required for tumor growth, blocking antibodies or small molecule inhibitors could be used for targeting.

Another approach based on the identity of a CD15-associated protein would be the generation of transgenic mice that express or respond to a toxin specifically in CD15+ cells based on the promoter for the specific CD15-associated protein. For example, mice containing lox-stop-lox Diptheria toxin receptor could be crossed with mice that express Cre recombinase under the control of the CD15-associated protein promoter (Brockschnieder et al. 2006). Treatment of the resulting animals with diphtheria toxin would then enable specific targeting of the cells that express the CD15-associated protein and assessment of the effects of killing TPCs within endogenous tumors.
Although knowledge of the CD15 carrier molecule may facilitate the development of novel targeting approaches, strategies to target these cells can also be developed without this knowledge. For example, the identification of pathways that are active specifically in CD15+ cells could also suggest potential avenues for targeting. Further studies that continue to elucidate the mechanisms that confer tumorigenic capacity to the CD15+ cells and assess the requirement of the cells for tumor growth may ultimately lead to improved strategies for the treatment of patients with medulloblastoma.

### 5.3 Targeting CD15+ cells through inhibition of G2/M cell cycle regulators

Rather than targeting the CD15-associated molecule or epitope itself, another strategy for targeting TPCs in patched mutant tumors involves understanding the unique properties and potential vulnerabilities of the CD15+ cells. As described in Chapter 4, we have shown that the CD15+ population contains an increased proportion of cells in G2/M phase of the cell cycle and corresponding elevated expression of Aurora and Polo-like kinases, important regulators of G2/M progression. This increased proportion of cells in G2/M was shown to be a vulnerability of CD15+ cells, as small molecule-mediated inhibition of Aurora or Polo-like kinases caused cell cycle arrest, enhanced the sensitivity of tumor cells to chemotherapy, and blocked tumor growth both in vitro and in vivo. Importantly, although these studies were initially based on targeting a property of tumor-propagating cells in the patched mutant mouse model, cells from patient-
derived xenografts of primary SHH-associated medulloblastoma were also sensitive to these agents. While these studies suggest promising potential for the use of these agents in the clinical treatment of medulloblastoma, important questions remain.

One question raised by our studies is why CD15+ cells progress more rapidly than CD15- cells through the cell cycle. Shh pathway activity has been shown to promote G1-S phase progression, thus differences in levels of Shh pathway activation between CD15+ and CD15- cells may contribute to the differences in cell cycle kinetics (Adolphe et al. 2006) (Kenney and Rowitch 2000) (Oliver et al. 2003). Previous studies from our lab have demonstrated that both CD15+ and CD15- cells have lost both alleles of patched, so both populations contain constitutive Shh pathway activation (Read et al. 2009). However, the activity of pathways that may cooperate with the Shh pathway (either through amplification of signaling through the Shh pathway itself or synergy with alternate pathways) to promote cell cycle progression may differ between CD15+ and CD15- cells. For example, CD15+ cells may have acquired additional alterations during tumorigenesis that promote proliferation. Alternatively, CD15+ and CD15- cells may be exposed to differing exogenous factors that cooperate with the Shh pathway, perhaps through residency in distinct microenvironments. CD15- populations in patched mutant tumors also display elevated levels of markers of differentiation and contain fewer actively cycling cells, but why these cells are more likely to differentiate while
CD15+ continue to proliferate remains unknown. Further studies will be required to determine the mechanistic basis for these differences between CD15+ and CD15- cells.

Our data demonstrating the sensitivity of mouse and human medulloblastoma cells to inhibitors of G2/M phase suggest that these agents may represent an approach to improve patient treatment. However, one practical question surrounding the use of Aurora or Polo-like kinase inhibitors in MB therapy is whether these molecules can penetrate the brain. The blood-brain barrier represents a significant obstacle for the delivery of many therapeutic agents, and our preliminary data (not shown) suggest that the Aurora kinase inhibitors VX-680 or PHA-739358 cannot cross this barrier upon systemic administration.

To determine whether other Aurora kinase or Polo-like kinase inhibitors have potential clinical utility, it will be important to assess the levels of the compounds within intracranial tumors after systemic delivery. However, an inability to penetrate the brain does not necessarily indicate that these agents do not have clinical utility. Other approaches for delivering non-penetrant agents to the brain have been developed, including convection-enhanced delivery and nanoparticle-mediated delivery. In convection-enhanced delivery, a catheter is placed in the proximity of the tumor after surgical removal of the tumor mass, and the agent is pumped into this local site at a constant rate of flow (Vogelbaum and Iannotti 2012). Alternatively, nanoparticles (coupled to the therapeutic agent) that have a propensity for bypassing the blood-brain
barrier and/or homing to the tumor can be generated and delivered systemically (Gagliardi et al. 2012). If the Aurora and Polo-like kinase inhibitors have poor brain penetrance, these approaches could be examined in our mouse models to determine if compound delivery can be improved and enable inhibition of tumor growth within the brain.

If delivery of Aurora or Polo-like kinase inhibitors to intracranial brain tumors proves to be challenging, another approach for targeting this vulnerability could be the use of inhibitors of other regulators of G2/M progression, such as Survivin or CDK1 (Mita et al. 2008) (Hirai et al. 2005). Although we focused on Aurora and Polo-like kinase inhibitors in our studies, the expression of many other G2/M regulators is also elevated in CD15+ cells (Read et al. 2009). Because we believe that this elevated expression is a result of increased residency in G2/M phase (rather than elevated expression of individual genes driving tumor growth), targeting any of multiple G2/M regulators could likely be equally effective at inhibiting tumor growth. If molecules that have been demonstrated to cross the blood-brain barrier are available for other G2/M regulators, these molecules could potentially be used (after pre-clinical assessment) as alternatives for MB therapy.

The studies described here were undertaken with the hypothesis that targeting tumor-propagating cells will improve cancer treatment by preventing therapeutic resistance or recurrence. While many studies (including ours) have provided in vitro
evidence that targeting tumor-propagating cells may enhance therapy, the question of whether killing these cells in endogenous tumors can truly fully eliminate the tumor’s capacity for recurrence requires further examination.

Our studies and others have shown that isolation of distinct populations from non-manipulated tumors that are capable of propagating the tumor is clearly possible. However, this population may not necessarily be stable over the course of tumor evolution, particularly during states of stress (such as upon treatment with cytotoxic agents). Selective pressure generated by therapeutic agents may lead to changes in the identity of the tumor-propagating population, such that the original population with this capacity is no longer necessary, but another population is capable of tumor regrowth. Therefore, strategies that target only the initially identified tumor-propagating may not necessarily be effective in the long run, as initially non-propagating cells may acquire this capacity. Strategies that instead target both the propagating and non-propagating populations may represent the ideal approach for preventing tumor recurrence.

Although this notion of targeting both the propagating and non-propagating populations may be reminiscent of the historical approach of treating the tumor bulk, past treatment strategies have not taken the often small subpopulations of tumor-propagating cells into account. Strategies that instead recognize both the capacity of tumor-propagating cells in therapeutic resistance as well as the potential for non-
propagating cells to acquire this capacity may be optimally effective. Fortunately, the approach of targeting MB tumor-propagating cells described here may also be effective in targeting the non-propagating population. Because Aurora and Polo-like kinases are generally required for progression through the cell cycle, any proliferative cell (regardless of its initial identity as propagating or non-propagating) may be sensitive to inhibition. Therefore, treatment with these inhibitors may represent an optimal approach for the treatment of MB.

### 5.4 Concluding statement

Together, these studies represent our efforts to advance our understanding of normal developmental mechanisms and the deregulation that occurs in malignant disease. By clarifying the role of CXCR4 signaling in postnatal cerebellar development, we have advanced our understanding of the factors that regulate the fundamental processes of proliferation, migration, and differentiation in the cerebellum, and this understanding will enable further studies to continue to expose the relationships between the signals involved in normal developmental and tumorigenesis.

Our studies of CD15+ tumor-propagating cells in patched mutant tumors have also advanced our knowledge of the properties and vulnerabilities of cells that may be responsible for tumor recurrence in patients. By recognizing the distinct cell cycle kinetics of CD15+ TPCs, we were able to identify regulators of G2/M phase progression as targets for therapeutic intervention. The ability of clinically available inhibitors of the
Aurora and Polo-like kinases to block the growth of both mouse and human medulloblastomas suggests that these agents may hold promising potential to improve treatment for patients. Future work that continues to examine the efficacy of these agents as well as alternative strategies for targeting tumor-propagating cells will hopefully ultimately enable patients diagnosed with medulloblastoma to survive and enjoy long and happy lives.
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Biography

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