MYC-driven Medulloblastoma

New Targeted Therapies and Mechanisms of Recurrence

ANNA BORGENVIK
Abstract

Medulloblastoma is the most common malignant brain tumor of childhood. It arises in the posterior fossa but presents with distinct histological and molecular features. Hence, medulloblastoma is divided into four molecular subgroups, WNT, SHH, Group 3, and Group 4. The overall 5-year survival is ~70% across subgroups but varies with high- and low-risk disease. Standard treatment of medulloblastoma consists of maximal safe tumor resection, radiotherapy, and adjuvant chemotherapy. Despite the rather high success rate of treatment for many patients it also comes with severe long-term debilitating side effects.

MYC proteins are master regulators of gene expression often deregulated in cancer. MYC family members MYC and MYCN share similar roles and are found overexpressed or amplified in most medulloblastoma subgroups and correlate with a poor prognosis.

Medulloblastoma dissemination and recurrence patterns differ between subgroups but are always associated with a poor prognosis. Recurrent medulloblastoma is not yet curable and will lead to death.

In this thesis, we present the first transgenic mouse model of medulloblastoma recurrence and highlight the role of the transcription factor SOX9 in MYC-driven relapse mechanisms. By studying this recurrence model and matched primary-recurrent patient samples we propose a mechanism in which treatment-refractory and quiescent SOX9-positive cells in Group 3 medulloblastoma are necessary for tumor relapse, and how the recurrent tumors can be specifically treated with MGMT inhibitors and doxorubicin.

In addition, we address efficient treatment options of primary MYC-driven medulloblastoma where BET bromodomain inhibition (JQ1) in combination with CDK2 inhibition (milciclib) of human Group 3 medulloblastoma will lead to apoptosis and prolonged survival of xenografted mice. This is explained by a dual hit on MYC transcriptional output and MYC protein stability exerted by JQ1 and milciclib respectively. Finally, in a different novel transgenic model of MYC-driven medulloblastoma, we show how temporal Cdk2 knock-out has no effect on MYC protein stability but slows down proliferation and prolongs survival of allografted mice.

The need for better treatments and increased understanding of recurrent medulloblastoma is huge. To that end, this thesis focuses on and addresses novel treatments, the role of the cell cycle protein CDK2 as well as relapse mechanisms depending on dormant SOX9-positive cells in highly aggressive MYC-driven medulloblastoma.

Keywords: Medulloblastoma, Pediatric cancer, Recurrence, MYC, MYCN, SOX9, Treatment, Bet bromodomains, Cyclin-dependent kinases

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


#,# Equal author contribution

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Other Papers by the Author

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<th>Description</th>
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<tbody>
<tr>
<td>BBB</td>
<td>Blood-brain-barrier</td>
</tr>
<tr>
<td>BET</td>
<td>Bromodomains and extra-terminal domain proteins</td>
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<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
</tr>
<tr>
<td>CSI</td>
<td>Cranio-spinal irradiation</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>Beta-catenin</td>
</tr>
<tr>
<td>D/N</td>
<td>Desmoplastic/nodular</td>
</tr>
<tr>
<td>DOX (dox)</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>E-Box</td>
<td>Enhancer box</td>
</tr>
<tr>
<td>E2F</td>
<td>E2 factor</td>
</tr>
<tr>
<td>EGL</td>
<td>External germinal layer</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signaling-regulated kinase</td>
</tr>
<tr>
<td>ERT2</td>
<td>Tamoxifen-inducible estrogen receptor</td>
</tr>
<tr>
<td>FBW7</td>
<td>F-box and WD repeat domain-containing 7</td>
</tr>
<tr>
<td>G1</td>
<td>Gap-phase 1</td>
</tr>
<tr>
<td>G2</td>
<td>Gap-phase 2</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
</tr>
<tr>
<td>GEMM</td>
<td>Genetically engineered mouse model</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GLI2</td>
<td>Glioma-associated oncogene homolog 2</td>
</tr>
<tr>
<td>Glt1</td>
<td>Glutamate transporter 1</td>
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<tr>
<td>GNP</td>
<td>Granule neuron precursor</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HMG</td>
<td>High mobility group</td>
</tr>
<tr>
<td>IGL</td>
<td>Internal granule layer</td>
</tr>
<tr>
<td>INK4</td>
<td>Inhibitors of CDK4</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LCA</td>
<td>Large cell/anaplastic</td>
</tr>
<tr>
<td>Luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>M</td>
<td>M-phase</td>
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Introduction

Cancer

In the latest cancer report from the World Health Organization (WHO), it was estimated that cancer is responsible for 8.2 million deaths worldwide\(^1\). In the same year, 14.1 million new cases of cancer were diagnosed and the incidence of cancer has generally increased over the past decades\(^1\). Fortunately, the mortality rate has decreased which is most likely explained by better diagnostic tools, new treatment regimens and, better general health care across the globe. The five most common sites of cancer among men are lung, prostate, colorectum, stomach, and liver, in order of incidence. The same data for women are slightly different and includes breast, colorectum, lung, cervix, and stomach\(^1\). In children, central nervous system (CNS) tumors are the most common cancer type followed by leukemia\(^2\), and the primary cancer-related cause of death\(^2,3\).

Brain tumors account for 2% of all cancer cases. The most common malignant brain tumor in adults, glioblastoma (GBM), has a 5% 5-year survival with a median survival of approximately 15 months\(^2,4\). Strong efforts have been made to improve the outcome for patients with GBM, but so far none have been curative. Medulloblastoma (MB), the most common malignant pediatric brain tumor has an overall survival (OS) of 70% that varies with subgroups\(^1,5\). The need for better and stratified treatments is huge also in this patient group. More than 50 different entities of tumors of the nervous system have been classified by the WHO. The complexity is enormous and stresses the notion that cancer is not a single disease but many.

Cancer develops from normal cells and tissues that acquire a cancerous phenotype. This might happen due to exposure to harmful environmental stressors such as UV light and certain chemicals, viruses, or familial genetic traits. It might also occur spontaneously\(^1\). In 2000 Hanahan et al. summarized the consensus regarding cancer cell characteristics and acquired features needed for tumor development, growth, and dissemination. In 2011 the old consensus was revised and two emerging hallmarks of cancer were added\(^6\). This thesis is largely aimed at exploring several aspects of cancer hallmarks centered around the pediatric brain tumor medulloblastoma. The data presented and discussed in the included papers relate to targeting highly proliferative tumor cells by attacking multiple important players involved in proliferation, growth, and cell death. Also, we explore how an interplay between
proliferation and quiescence led to tumor cell invasion, metastasis, and in the end, relapse. In addition to this, we present evidence of tumor cell immune escape. Following is a brief summary of the hallmarks of cancer.

**Sustained Proliferative Signaling**
Cancer cells can derail the normal control of proliferation. This can be achieved through many mechanisms. Cancer cells can stimulate their proliferation through autocrine signaling, upregulation of surface receptors. Or, removing the ligand from the equation by either structurally change the receptor to signal even in the absence of ligand or activate downstream proteins completely independently of receptor signaling. Strong oncogenic signaling from MYC and RAS stimulates proliferation but is a balancing act in which high levels cause apoptotic or senescent responses. The key is to keep the levels just enough to escape those fates or derail the mechanisms controlling them⁶.

**Evading Growth Suppressors**
Two major gatekeepers of growth and proliferation must be circumvented to sustain uncontrolled cell proliferation. The most famous examples include the tumor suppressors pRb (retinoblastoma protein) and p53. pRb generally regulates cell cycle progression based on extracellular signaling while p53 senses signals of distress from inside the cell (such as DNA damage, oxygen levels, and building blocks for cell division). Furthermore, the proliferative repressor mechanism of contact inhibition is lost in cancer cells due to the loss of cell surface adhesion molecules and epithelial integrity⁶.

**Resisting Cell Death**
The most common mechanism behind resistance to cell death in cancer cells is the loss of p53. There might also be upregulation of anti-apoptotic proteins or downregulation of other pro-apoptotic proteins than p53 in the cell death machinery. Autophagy is the mechanism through which cells can degrade organelles for the recycling of catabolites and works together with apoptosis to maintain homeostasis. It is believed to be an additional mechanism for sustained growth, proliferation, and cell death escape. In addition, necrosis might stimulate tumor progression by releasing pro-inflammatory signals beneficial for angiogenesis, proliferation, and invasiveness. This is in contrast to the general idea that cancer cell death is beneficial for tumor regression⁶.

**Enabling Replicative Immortality**
Normal cells undergo a limited number of replications before they enter a non-proliferative state, known as the Hayflick limit or replicative senescence. This is mainly controlled by the shortening of telomeric DNA with each cell division and eventually leads to senescence or apoptosis. Cancer cells, on the other hand, can divide indefinitely. Telomerase adds telomere repeat segments to DNA and is expressed in human cancer cells. Increased activity of telomerase,
e.g. by point mutations in the promoter of the telomerase gene, counteract the shortening of telomeric DNA and the cancer cell becomes immortalized\textsuperscript{6}.

**Inducing Angiogenesis**

Like all cells, cancer cells need oxygen and nutrients to thrive. New vessel formation (angiogenesis) is normally restricted to specific physiological events such as wound healing and the menstrual cycle. However, during tumor development the angiogenic switch is activated and sustained to cause vessel sprouting, forming new vessels that supply the tumor with nutrients. These tumor vessels are often abnormal in all aspects of structure and function\textsuperscript{6}.

**Activating Invasion and Metastasis**

The process of metastasis generally includes local invasion, intravasation into blood or lymph, transfer through vessels, extravasation from vessels, micrometastasis, and lastly colonization. The biological changes in the cell required for this cascade of events might be almost fully explained by epithelial-mesenchymal transition (EMT). EMT naturally occurs during development and the various transcription factors (TFs) governing the process are again upregulated in many cancers. The microenvironment has shown to be of importance for the cancer cells’ ability to migrate\textsuperscript{6}.

**Reprogramming Energy Metabolism**

Compared to normal cells, cancer cells prefer to harvest energy mainly through glycolysis despite the aerobic environment. This process is not as efficient as normal glucose metabolism under aerobic conditions where energy is sequestered from mitochondrial oxidative phosphorylation. Cancer cells sustain their metabolic switch by upregulating glucose transporters to substantially increase glucose levels in the cell. This might be important for highly proliferating cells that need a lot of building blocks to sustain the proliferative rate\textsuperscript{6}.

**Evading Immune Destruction**

By studying tumor formation in immune-deficient mice, it has become evident that both innate and adaptive immune systems are important for immune surveillance of tumors and the eradication of such. Less is known about this emerging hallmark but studies have shown that tumor cells may secrete immune suppressive factors to attenuate the immunogenic response. The recent explosion in successful immunotherapies, treatments that restore or enhance the immune system's ability to fight cancer, emphasizes the importance of this hallmark\textsuperscript{6}.
Medulloblastoma

Molecular and histological classification

Medulloblastomas are classified as WHO high-grade (IV) tumors and constitute 25% of all pediatric intracranial neoplasms making them the most common entity of malignant pediatric brain tumors. The overall incidence is ~6 cases per 1 million children\(^2\). 77% of patients are under the age of 19 at diagnosis and incidence peaks at 3 and 7 years of age. The disease is more common in males than in females\(^2\). MB can present with different histological patterns such as classic (undifferentiated round small cells), desmoplastic/nodular (round pale nodules of reticulin-free zones), MB with extensive nodularity (MBEN, larger lobules of reticulin-free zones), and large cell/anaplastic (undifferentiated, pleomorphic cells with large nuclei)\(^5\). Molecular subgrouping based on gene expression profiling has resulted in the division of MB into four distinct subgroups; WNT (Wingless), SHH (Sonic hedgehog), Group 3, and Group 4\(^7\)-\(^9\). Named after prominent signaling pathways involved, Group 3 and Group 4 were ambiguous on this matter with no obvious molecular signature driving tumor development. Early on, when the first genetically based classifications were published\(^10\)-\(^14\) and later summarized in a consensus paper\(^7\), it was clear that there were further depths to explore within the four subgroups\(^7\),\(^15\). Recent efforts have been made to achieve a better understanding of intertumoral heterogeneity within all subgroups. In 2017, two publications made major contributions to the understanding of this. Cavalli et al.\(^16\) used Similarity Network Fusion to combine genome-wide DNA methylation data and gene expression of 763 MB samples. They revealed new levels of subtypes within the four subgroups. Using large cohorts of genome, epigenome, and transcriptome data Northcott et al. successfully identified novel drivers of disease within all MB subgroups. Notably, potential driver events of large portions of Group 3 and 4 tumors were found\(^17\). Another similar paper was also published that same year based on a smaller cohort of tumors but was focused more on risk stratification with potential for clinical use in drug trials\(^18\). These findings are further discussed below and summarized in figure 1.
Figure 1. General overview of MB molecular subgroups and associated features.

**WNT medulloblastoma**

Patients with WNT-activated MB have an excellent prognosis (close to 100% OS) when given appropriate treatment\textsuperscript{19}. The subgroup accounts for 10% of all MBs and affects older children and adults\textsuperscript{16}. MBs in this subgroup have almost exclusively classic histology\textsuperscript{5}. WNT tumors are believed to arise from cells in the lower rhombic lip of the dorsal brain stem. However, these tumors are found in the cerebellar midline, not always in close proximity of the brain stem\textsuperscript{20, 21}. Interestingly, methylation profiling of a small cohort of pineoblastomas identified them as WNT MBs\textsuperscript{22}.

As the subgroup name entails, WNT MBs have WNT signaling pathway activation and are most often characterized by monosomy 6 (83% of WNT MBs)\textsuperscript{17} and/or \textit{CTNNB1} mutation (86% of WNT MBs)\textsuperscript{8, 17, 23}. Recently, two subtypes of WNT MB (\(\alpha\) and \(\beta\)) were recognized primarily based on the age of the patient, with children and adolescents in \(\alpha\) and adults in \(\beta\)\textsuperscript{16}. Monosomy 6 is found in almost all WNT \(\alpha\) tumors, but not all within the \(\beta\) subtype\textsuperscript{16}. 
However common, neither \textit{CTNNB1} mutation nor monosomy 6 are found in all WNT MBs. This points to the necessity of finding additional markers not to misdiagnose patients. Other genetic aberrations have been identified and found to be rather common amongst WNT-activated MBs. These are somatic mutations in \textit{DDX3X}, \textit{SMARCA4}, and \textit{TP53} and, \textit{APC} germline mutations in patients with Turcot syndrome \textsuperscript{24, 25}.

\textbf{SHH medulloblastoma}

SHH-activated MB most likely arises from granule neuron precursors (GNPs) in the developing cerebellum\textsuperscript{26, 27}. During normal cerebellar development, GNPs in the external germinal layer (EGL) are dependent on SHH signaling to proliferate. This is achieved by the secretion of SHH ligand from Purkinje neurons also found in the developing cerebellum. Loss of inhibitors and gain of function mutations in this crucial pathway causes abnormal activation of GNPs and disease\textsuperscript{28}. SHH tumors occur in all age groups\textsuperscript{16} and account for \~30% of MBs\textsuperscript{16}. SHH-activated MB can present with different histological features, foremost desmoplastic/nodular and MBEN\textsuperscript{17} but also classic or large cell/anaplastic (LCA) morphology. Histology seems to be a part of the intertumoral heterogeneity of SHH MB\textsuperscript{5, 16}.

In the 2016 update of the WHO MB classification, the SHH subgroup is further divided based on \textit{TP53} mutational status (\textit{TP53}-wildtype/ \textit{TP53}-mutant)\textsuperscript{5}. Also, the common genetic aberrations associated with this subgroup differ based on \textit{TP53} status. The most common are \textit{TP53}-wildtype tumors\textsuperscript{17}. They are associated with germline (Gorlin syndrome\textsuperscript{29}) or somatic mutations in \textit{PTCH1} or \textit{SUFU}, \textit{SMO}, and rarely \textit{GLI2} causing activation of SHH signaling\textsuperscript{5, 25}. \textit{TP53} mutation, found in 13% of SHH MBs\textsuperscript{17}, is, however, associated with amplification of \textit{GLI2}, \textit{MYCN}, or \textit{SMO} leading to SHH pathway activation\textsuperscript{5}. Cavalli et al. discerned four subtypes of SHH MB (\(\alpha\), \(\beta\), \(\gamma\), \(\delta\))\textsuperscript{16}. \textit{TP53} mutation, \textit{MYCN}, and \textit{GLI2} amplifications were most commonly found in SHH\(\alpha\) and has the worst prognosis of all SHH subtypes\textsuperscript{16}. Similar to what has been previously reported\textsuperscript{5}. Other factors such as age at diagnosis, prognosis, and \textit{TERT} promoter mutations also sets the subtypes apart\textsuperscript{16}. Recently it was reported that expression of non-coding RNAs is SHH subtype specific\textsuperscript{30}. However, deciphering their role is difficult due to poor annotation but pathway analysis suggests that they might play an important role. In addition, the authors report several novel genes associated with Cavalli SHH subtypes such as \textit{PPM1D}, \textit{GNAS}, \textit{ZBTB20}, and \textit{IKBKAP}\textsuperscript{30}.

\textbf{Group 3 and 4 medulloblastoma}

Group 3 and 4 MB are defined as non-WNT/non-SHH tumors (according to WHO), distinct based on transcriptome, methylome, and microRNA data\textsuperscript{3}. Prominent signaling pathways that drive and separate these two subgroups are not obvious and have been hard to identify due to high intertumoral heterogeneity. However, the understanding of Group 3 and 4 MB has increased
substantially due to the recent publications looking at large data sets of MB tumors. It has been suggested that several subtypes of Group 3 and 4 MB exist. While Northcott et al. identified up to eight subtypes in a collection of Group 3 and 4 tumors (I-VIII)\textsuperscript{17}, Cavalli et al. recognized three subtypes of each (α, β, γ)\textsuperscript{16}.

Group 3 MB is affecting infants and children but rarely adults and makes up ~20% of all MB cases\textsuperscript{7,15,16}. It presents largely with classic or LCA histology\textsuperscript{7}. Mutations or amplification of MYC, GFI1B, SMARCA4, KBTBD4, MYCN, and OTX2\textsuperscript{17} are known common genetic features of Group 3 MBs. MYC amplifications are found in 17% of Group 3 MB\textsuperscript{17}. With the addition of the relatively rare cases with MYCN amplification (5%)\textsuperscript{17,31}, MYC/MYCN amplification/overexpression constitutes one of the most important drivers in this subgroup\textsuperscript{17}. MYC/MYCN overexpression is correlated with a poor prognosis in general for MBs\textsuperscript{15} which is reflected in both Group 3 and 4 tumors.

Group 4 tumors constitute the majority of MB cases (~40%), displaying classic histology and affects children between 5-15 years of age\textsuperscript{7,15,16}. The most prominent molecular feature of Group 4 MB is copy number alterations of chromosome 17 (17q gain or most commonly isochromosome 17q with loss or 17p and duplication of 17q) that occur in about 80% of these tumors and across subtypes\textsuperscript{16,32}. Mutation and/or amplification of PRDM6, GFI1B, KDM6A, OTX2, ZMYM3, MYCN, and CDK6 are seen in order of frequency\textsuperscript{17}. GFI1 and GFI1B have emerged as important drivers in subsets of Group 3 and 4, with overexpression/amplification in 15% of Group 3 and 12% of Group 4 tumors\textsuperscript{17,33}. These oncogenes are activated mutually exclusively through enhancer hijacking caused by structural variants positioning these genes close to enhancer elements\textsuperscript{33}. The same mechanism seems to also activate translation of the relatively unknown PRDM6 protein through a SNCAIP enhancer element, but only in Group 4\textsuperscript{17}. In addition, the ubiquitin ligase adaptor protein (KBTBD4) is a recently suggested driver of both subgroups. Different in-frame insertions in KBTBD4 occur in Group 3 and 4 that further cluster with two distinct subtypes\textsuperscript{17}.

The cell of origin for Group 3 and 4 MB is still largely unknown. A long-standing hypothesis is that Group 3 tumors arise from neural stem cells in the developing cerebellum\textsuperscript{34}. Single-cell transcriptomics has recently supported this and additionally proposed unipolar brush cells as the cell of origin for Group 4 MB\textsuperscript{35}.

**Diagnosis and current treatment of medulloblastoma**

MB patients have their diagnosis after evaluation of their clinical symptoms, magnetic resonance imaging (MRI) of brain and spine, and further analysis of cerebral spinal fluid (CSF) to assess the primary tumor as well as to detect metastases\textsuperscript{36}. In addition, both histopathological and molecular analyses are used\textsuperscript{36}. Following diagnosis, the standard treatment regimen of MB includes
tumor resection, radiotherapy (craniospinal irradiation (CSI) and tumor site boost), and chemotherapy that increases survival of high-risk MB to 50-60% and average risk to 80-90% \cite{37,38,39}.

First and foremost, patients undergo surgery to resect as much of the tumor as possible. At this point, a specimen of the tumor is extracted for histopathological and genetic/molecular analysis, a crucial part of diagnosis \cite{36}. It is standard to use a combination of these for diagnosis based on the WHO definitions of MB \cite{5}, when possible. DNA methylation is the preferred method for clinical subgrouping/subtyping of MB. It is further important to evaluate TP53 mutational status as well as MYC/MYCN amplification as these have major effects on patient risk stratification in SHH, Group 3 and 4 MB, respectively \cite{36}. This is followed by radiotherapy and adjuvant chemotherapy. Due to the severe side effects of irradiation to the developing CNS, children under 3-5 years of age are excluded from this treatment \cite{36,40}. Older patients are treated with CSI at different doses (15-36 Gy) \cite{36} depending on the risk stratification at diagnosis. These patients also receive a focal boost of irradiation to the tumor site (total dose: 54 Gy) \cite{36,40}. Additive to surgery, irradiation of MBs has proven to be the most successful of all treatment options currently available \cite{41,42}. Newer technology has allowed a decrease in non-target tissue exposure with the potential of lowering the adverse effects of radiotherapy as reviewed in \cite{40}.

All patients receive adjuvant chemotherapy. This is of utmost importance for young patients that are not eligible for irradiation. Despite adjustments (such as the addition of methotrexate) specifically for this group of patients, their outcome is still not improved (SJYC07; NCT00602667) \cite{43}. The most commonly used chemotherapeutics for MB are cisplatin, carboplatin, vincristine, cyclophosphamide, and lomustine \cite{36}.

Most patients are enrolled in clinical trials and further stratified based on high/low/intermediate-risk into a cycling treatment regimen including multi-agent combinations of the drugs mentioned. It is proven hard to include newer and targeted therapies into the standard treatment of medulloblastoma. Some of these have shown inferior results in early clinical trials and some, such as SMO inhibitors for the SHH subgroup, lead to resistance \cite{44}. Better and perhaps more specific stratification based on molecular signatures could hopefully help in finding targeted drugs that will benefit a specific group of patients. Now, promising effects for targeted therapies might be diluted in clinical trials with suboptimal stratification and wide inclusion criteria. This is further complicated by the rarity of these brain tumors making treatment groups small. There is a great need to better understand MB mechanisms to find novel targeted therapies that not only cure the patient but also safeguard these children’s normal development and long-term quality of life.
Medulloblastoma metastasis and recurrence

Medulloblastoma is graded as M0-M4 based on the metastatic status at diagnosis inferred from MRI of brain and spine as well as CSF cytology. The grading ranges from no metastases (M0), micrometastases in the CSF (M1), gross nodular seeding intracranially beyond the primary site (M2), gross nodular seeding in the spinal cord (M3), to metastases found outside of the CNS (M4). Metastatic dissemination in MB occurs primarily via leptomeningeal spread and stays within the CNS. There is also evidence of how MB tumor cells can circulate in the bloodstream and cause leptomeningeal metastases. All MB subgroups can present with metastases at diagnosis but it is rarely seen in WNT and SHH MB, except for adult SHH tumors (SHHb) that has a higher metastatic frequency. Group 3 and 4 MB are rather homogeneous when it comes to their high metastatic status, with ~40% having metastases at diagnosis. However, in the Cavalli subtype 3β, the metastatic frequency is lower, around 20%. Subgroup-specific differences in the location of brain metastases have been reported with Group 4 specific lesions in the anterior part of the third ventricle. Molecular drivers of MB metastasis have been investigated and are reviewed in. Pathways such as RAS/MAPK, PI3K, HGF/cMET, and NOTCH are implicated in metastasis mechanisms.

Not all MBs recur. But when they do, they stay within their respective subgroup, and most often within the CNS but are unfortunately almost always lethal. Recurrences occur in all subgroups however less common in WNT MB. SHH MB generally recurs locally in the primary tumor bed while Group 3 and 4 have metastatic recurrences at distant locations. Despite their unchanged subgroup affiliation at relapse massive clonal evolution is reported at recurrence compared with treatment naïve primary tumors. Not much is known of the mechanisms behind MB recurrences. There are publications implicating an upregulation of PDGFRA, SOX2 positive cells in SHH MB, and MYC/MYCN amplification together with TP53 loss to be involved in MB recurrence. Group 3 and 4 tumors’ metastatic signature render patient survival low. Therefore, new measures must be taken to develop better models of recurrence. Such models can be utilized to find better treatments that not only target the primary tumor but also the more resistant cells in the tumor bed and distant metastases. In paper II we have made an effort towards this end by developing the first mouse model of recurrent MB based on the already existing Group 3 mouse model GTML.

Models of medulloblastoma

Tumor cell biology and new treatments are most easily studied using cell cultures in a petri dish. Cell culturing allows us to study tumor cells from various biological model systems as well as cells from human tumors. Under these relatively simple conditions, we can easily evaluate gene modifications, both
large- and small-scale drug screens as well as drug concentration evaluation for further studies in more complex systems and more. This is also true for in vitro MB tumor modeling for which there exist several cell lines. Some of these are cultured as monolayers while others grow as neurospheres, adding another level of complexity to maintain tumor cell characteristics. Nevertheless, there are drawbacks to cell culture models. These cultures are subjected to early clonal selection and will change with passaging. Therefore, it is preferred to use primary cell cultures from humans or mice as compared to established cell lines that perhaps no longer represent the disease they came from. Unfortunately, not all MB subgroups are easily studied in cell culturing conditions due to a lack of cell lines or culturing difficulties. No matter how hard we try, monolayer cell culturing will never recapitulate the complexity of the tumor being modeled nor the microenvironment that is proving to be more and more important for tumor maintenance\textsuperscript{58}. More advanced techniques of cell culturing have emerged. Cells can be cultured in three dimensions using matrix proteins to form organoids that better represent normal conditions\textsuperscript{59}. Indeed Group 3 MB is modeled in an organoid system\textsuperscript{60}. To bridge the gap between petri dish and patient, researchers use simpler organisms to study tumorigenesis in a complex biological system. Mice have been used for this purpose due to their small size (making them cheap to host compared to larger animals), biological complexity, fast breeding time, and the many tools that are available for mouse genome editing. The ideal model would manifest the same clinical, biological, genetic, and histological features as the human disease and be a good representative of treatment studies for optimal translational purposes. Unfortunately for MB, mouse models still cannot recapitulate human tumor complexity completely. This is in part explained by oncogenic drivers in mouse models that are not presented in human disease and species differences in orthologs. Therefore, knowledge of the limitations and benefits of the available models is crucial to pick the most relevant for the intended study, and/or develop new mouse models that better represent the disease. Many techniques can be used and even combined in complex systems to establish a mouse model. Some of these and the current mouse models of MB are discussed below.

**Xenograft models**  
Human tumor cells can be transplanted into immunocompromised mice either at the corresponding original site of the tumor (patient-derived orthotopic xenograft, PDOX) or a different site (patient-derived heterotopic xenograft, PDX). Preferably, the cells have not been in culture before transplantation or in between passaging from mouse to mouse. In this way, secondary tumors that recapitulate the primary tumors are formed and can be studied in for example drug screens. There are drawbacks to these PDXs, including the lack of a representative microenvironment in immunocompromised mice. Also, loss of an intact blood-brain barrier (BBB) has been observed in PDXs (not
published data). The reason for this is not known but might be due to the invasive transplantation disrupting the BBB and/or the artificial tumor growth over a short time in an older animal. Importantly, this will compromise any treatment studies on all subgroups of MB except for WNT MB that inherently has a non-functioning BBB\textsuperscript{61}. Similar to cell culturing, also tumor cells in a PDX can change with passaging in mice.

Tumor cells extracted from mouse models can be transplanted into immunocompetent mice (allograft) to study tumor formation and treatment in a relevant microenvironment. However, these tumors share problems with the genetically engineered mouse models (GEMMs), the tumors will not completely represent their human counterparts.

**Conventional genetically engineered mouse models (GEMMs)**

*Tet-ON/OFF system*

The antibiotic tetracycline (Tet) or its more stable variant doxycycline (dox) can be used to reversibly turn on or off the expression of a gene of interest. A tetracycline controlled transactivator (tTa) is expressed from a promoter of choice (e.g., a tissue-specific promoter (TSP)) and binds to a Tet responsive element (TRE) containing a binding site (TetO) for tTA and a downstream minimal promoter (such as a CMV promoter) that will drive the expression of your gene (transgene, Tg) of interest upon tTA expression. In a *Tet-OFF* system, the absence of tetracycline or doxycycline will activate expression and turn it off when added (Fig. 2i). In a *Tet-ON* system, the presence of the antibiotic will activate the expression (Fig. 2ii)\textsuperscript{62}. Both the *Tet-ON* and *Tet-OFF* systems were utilized to create the mouse models in all three papers of this thesis. In papers I and III we used two similar mouse models of Group 3 MB. Both the GTML\textsuperscript{57} (in paper I) and GMYC\textsuperscript{63} (in paper III) were based on the *Tet-OFF* in which we drive expression of MYCN and MYC respectively from the *Glt1* promoter. MYCN/MYC can be turned off using dox to cure the mice\textsuperscript{57, 63}. In the GTML model, we can follow tumor development with a bidirectional expression of Luciferase and MYCN (Fig. 2i), something that we cannot do with the genetic setup of GMYC. In paper II, the *Tet-ON* and *Tet-OFF* systems were combined to create a recurrence model of Group 3 MB (Fig. 2). Without dox, primary tumors are formed based on the GTML model with MYCN expressed from the *Glt1* promoter (Fig. 2i). When the mice are then treated with dox, it generates a “*Tet-switch*” of MYCN expression from the *Glt1* promoter to the *Sox9* promoter; *Tet-OFF* to *Tet-ON* (Fig. 2ii).
**Figure 2.** Modified from paper II. Schematics showing the TetOFF (i) and TetON (ii) systems and how they can work in concert using a “Tet switch” with doxycycline (Dox) to promote MB relapse in the GTS model of MB recurrence.

**Cre recombinase-LoxP system**

To knock out or delete a gene of interest the Cre recombinase-LoxP technology can be used. LoxP sites are placed downstream and upstream of the gene of interest. These sites are recognized and bound to by a Cre recombinase leading to homologous recombination between the two loxP sites causing the genetic information in between to be deleted or “knocked out”. The system can be conditioned by controlling the recombinase activity usually by tamoxifen treatment. Cre-induced homologous recombination is used in paper III to knock out cyclin-dependent kinase 2 (Cdk2) in MYC-driven MB\(^63\). Here, we crossed GMYC mice with mice carrying Rosa26-CreERT2;Cdk2\(^{fl/fl}\) to generate a Group 3 MB mouse model in where we could knock out Cdk2 with tamoxifen-induced Cre.

**RCAS/tv-a**

Another important model system is the retrovirally mediated GEMM that takes advantage of the Replication Competent ALV Splice acceptor (RCAS). RCAS is a chicken retrovirus that requires the expression of an avian tv-a receptor to infect mammalian cells. This system is mostly used to target the delivery of a specific gene of interest to a specific cell population. For example, any oncogene can be incorporated into the RCAS vector and infect cells expressing a tv-a receptor linked to a tissue-specific promoter. Several tissue-specific vectors for brain tumor development are currently available, Ntv-a (nestin-positive cells)\(^64\), Gtv-a (GFAP positive cells; astrocytes and adult NSC)\(^65\), Ctv-a (CNP positive cells; oligodendrocytes and oligodendrocyte progenitors)\(^66\), and Olig2-tv-a\(^26\). Experimentally, mice positive for the tv-a gene are intracranially injected with the RCAS virus-carrying any oncogene of interest or with the chick cells (DF1) producing the virus. In this way, overexpression of a particular oncogene can be induced postnatally in specific cells.
Mouse models of WNT medulloblastoma
Only one mouse model of WNT MB exists. The model is a beta-catenin mutant in combination with loss of $T_p53$ ($CTNNB1$-mutant/$T_p53^{flx/flx}$)\textsuperscript{20}. However, only a small proportion of WNT MB harbor $TP53$ mutations. Also, monosomy 6, another hallmark of WNT medulloblastoma besides beta-catenin, is not modeled. New models covering the spectra of WNT subtypes could help lower treatment doses to maintain an excellent prognosis for these patients but also minimize unnecessary side effects.

Mouse models of SHH medulloblastoma
There are plenty of GEMMs representing SHH MB. Most of the models appear to be similar to adult SHH MB and the majority have an alteration in SHH signaling, reviewed in\textsuperscript{67, 68}. Unfortunately, few models that mimic infant or childhood human SHH MB driven by downstream effectors of SHH signaling are still lacking. Efforts in mutating $SUFU$ have been made but only give rise to tumors with loss of $T_p53$, an event unlikely to be seen in infant patients\textsuperscript{67}. Recently, members of our lab published a humanized mouse model of infant SHH MB, based on MYCN overexpression in primary human hindbrain-derived neuroepithelial stem cells or iPSC-derived neuroepithelial stem cells\textsuperscript{69}. Similar to this is the neuroepithelial stem cell models, derived from Gorlin patients\textsuperscript{70, 71}.

Mouse models of Group 3 and 4 medulloblastoma
Most mouse models of Group 3 MB are driven by MYC. The GTML model ($Glt1$-tTA;$TRE$-$MYCN$/Luciferase) is driven by wild-type MYCN overexpression from the $Glt1$ promoter in a Tet-OFF system\textsuperscript{57} as discussed earlier. Models that utilize wildtype (WT) or stabilized MYC\textsuperscript{T58A} have concomitant mutations of pro-apoptotic $TP53$\textsuperscript{34, 56, 72, 73} or overexpression of oncogenic $Gfi1$\textsuperscript{74}, anti-apoptotic Bcl2\textsuperscript{72}, or Otx2 (in an organoid model)\textsuperscript{60}. Interestingly, in the previously discussed GMYC model\textsuperscript{63} MYC alone is used as an oncogenic driver of Group 3 MB. Furthermore, loss of $Mll4$, a methyltransferase, was recently found to drive MB development in mice\textsuperscript{75}.

For a long time, there were no available mouse models of Group 4 MB. However, phosphoproteomics has revealed increased ERBB4-SRC signaling that could be used to model Group 4 MB. By combining constitutively active SRC with a dominant-negative p53 authors could generate the first in utero model of this subgroup\textsuperscript{76}. In addition, a xenograft model of Group 4 MB has been developed and shown to be regulated by BMP signaling for tumor volume expansion and invasion\textsuperscript{77, 78}. 
SOX9 and its role in cancer

SOX proteins belong to the high mobility group (HMG) box superfamily of transcription factors. More specifically, these proteins (SOX; SRY-related HMG-box) share a highly conserved HMG-box DNA binding domain of the SRY gene important for sex determination. There are 20 known SOX proteins divided into groups A-H, SOX9 belonging to group E, based on HMG-box sequence similarity. The transcriptional activity of SOX9 proteins is dependent on hetero- or homodimerization. SOX9 is primarily implicated in neural crest cell development and differentiation, chondrogenesis, and sex determination. SOX9 has also been shown to be required for neural stem cell development and maintenance in both embryonic and adult CNS. Gliogenesis in the developing brain and spinal cord is dependent on SOX9 transcriptional activity. Specifically, it is crucial for the switch from neurogenesis to initiation of gliogenesis. However, this regulatory capacity of SOX9 seems to be different in the cerebellum where SOX9 mediates cessation of neurogenesis but is not necessary for gliogenesis.

There is plenty of evidence for SOX9 involvement in cancer. SOX9 plays a role in multiple cancer types such as skin, prostate, lung, breast, and brain. And, it can function both as an oncogene and a tumor suppressor, seemingly context dependent. SOX9 has been implicated as a marker or driver of the cancer stem cell state as well as cell plasticity to promote metastasis, exemplified in lung cancer. SOX9 together with SLUG induces EMT in proliferating SOX2 positive cells, promoting invasion. A similar phenomenon has also been reported in melanoma. Moreover, SOX9-SLUG signaling is also shown to promote breast cancer metastasis. In addition, SOX9 has implications in treatment resistance towards e.g. cisplatin in MB, non-small-cell lung cancer, and hepatocellular carcinoma, temozolomide in glioma, and tamoxifen in breast cancer. A few specific examples further support the role of SOX9 in latent tumor cells that escape the immune system and promote metastasis or relapses in breast, lung, prostate, and skin cancer. The mechanism behind this SOX9-driven tumor latency is likely context-dependent but relies on either autocrine inhibition of WNT or involves SOX9-dependent resistance to specific immune cells.

SOX9 is regulated by the ubiquitin-proteasome system (UPS) and is a target of FBW7 activity (an E3 ubiquitin ligase) for its degradation. MB with FBW7 loss is strongly correlated with high SOX9 levels and is further linking SOX9 with the expression of genes promoting metastasis and treatment resistance. The importance of SOX9 in brain cancer and specifically GBM, and medulloblastoma is emerging. Much can be learned from what is known in other cancer types, with the caveat that the mechanisms seem to be largely context-dependent. Paper II is an effort to unravel a previously unknown mechanism of SOX9 in the context of tumor recurrence. In the
combined Tet-\textit{OFF}/Tet-\textit{ON} mouse model of recurrent MYC-driven MB (the GTS model) SOX9 is found to be necessary for tumor relapse.

**MYC protein family**

The family of MYC proteins has three members, MYC (c-MYC), MYCN, and MYCL. All of which are Basic Helix-Loop-Helix transcription factors\textsuperscript{114}. MYC is highly regulated by the UPS and a series of phosphorylations of specific residues for its turn-over (Fig. 3). Phosphorylation both activates and targets MYC proteins for degradation. MYC proteins are activated and stabilized through extracellular growth stimulatory signals that promote phosphorylation of serine 62 (S62) by ERK proteins or CDK1/2\textsuperscript{115-117}. MYC can then hetero-dimerize with its bHLH partner MAX to promote transcription and with MIZ1 to repress it\textsuperscript{118, 119}. pS62 is at the same time recognized by GSK3$\beta$ that phosphorylates threonine 58 (T58) proceeding de-phosphorylation of S62 and enables binding of FBW7 that targets MYC for proteasomal degradation\textsuperscript{115, 120} (Fig. 3). This renders the half-life of MYC proteins rather short, about 20 min\textsuperscript{121}.

MYC proteins, primarily MYC and MYCN, are pivotal in normal development exemplified by embryonal lethality in mice lacking these proteins\textsuperscript{122, 123}. MYCN plays an important part in especially cerebellar development. SHH signaling from Purkinje neurons in the EGL promotes MYCN expression in GNPs that start to proliferate and migrate to form the internal granule layer (IGL)\textsuperscript{124}. MYC proteins are strong oncogenes found amplified or overexpressed in many cancers including SHH, Group 3 and 4 MB\textsuperscript{15, 17, 124, 125}. As aforementioned, high MYC protein levels correlate with a poor prognosis\textsuperscript{15}. MYC and MYCN are mutually exclusive in these settings where MYCN overexpression and/or amplification is mostly seen in SHH and Group 4 MB and MYC in Group 3 and WNT subgroups\textsuperscript{17, 124}. 
Therapeutically targeting MYC

It is a well-known fact that transcription factors such as MYC proteins are hard to target. They lack the easily targetable structures proteins with enzymatic activity harbor. And, they have a fast turnover in cells making it difficult for any inhibiting drugs to have sustained effects\textsuperscript{126}. Also, MYC proteins’ importance for normal cell homeostasis increases the risks for side effects. In addition, MB with high MYC levels requires the potential MYC targeting drug to cross the BBB. Despite these hurdles, alternative modes of inhibition have shown potential and new ideas are constantly evolving with new knowledge. Instead of targeting the MYC protein itself, one can indirectly inhibit MYC expression, regulation, and/or output. For example, inhibit the dimerization with a MYC binding partner, block the transcriptional machinery on DNA or find easier targets such as upstream kinases important for MYC activity and degradation (discussed further in\textsuperscript{127}). In this thesis, I have studied two ways of indirectly inhibiting MYC in MB and combined them to maximize the therapeutic effect. The two modes of action are discussed below and further in papers I and III.

BET bromodomains as epigenetic drug targets

Epigenetic modification of the chromatin structure is important for controlling gene expression. Acetylation of lysine residues on histone (H) tails functions as binding sites for bromodomains and extra-terminal domain proteins (BET). There are four of these highly conserved epigenetic readers in humans: BRD2,
BRD3, BRD4, and BRDT. BRD4 is the most extensively studied and recognizes acetylated H3 and H4 for chromatin binding via its two bromodomains (BD1 and BD2)\textsuperscript{128}. BRD4 further binds the cyclin T1 domain of P-TEFb, a heterodimer kinase. The P-TEFb kinase domain, CDK9, will then be able to phosphorylate RNA polymerase II\textsuperscript{129, 130}. This complex machinery of transcriptional control will lead to subsequent transcriptional elongation. Interestingly, BRD4 is important for the expression of oncogenes such as MYC and its transcriptional output\textsuperscript{131}. Also, BET bromodomains are enriched at super-enhancer regions in several cancers\textsuperscript{132}. In 2010, the first proof-of-principle BET bromodomain inhibitors were published\textsuperscript{133, 134}. They have since shown promising and exciting results on MYC-driven cancer\textsuperscript{133-137}. The BRD4 inhibitor JQ1 competitively binds acetylation recognition sites and has shown good efficacy on MYC/MYCN-driven Group 3 and 4 MB\textsuperscript{133, 138}. Other BET inhibitors have emerged and been extensively studied in clinical trials\textsuperscript{139}. TEN-010 (JQ2) is currently in clinical trials (NCT02308761, NCT01987362). In paper I we used JQ1 to target tumor cells in our MYC-driven MB models. We also combined JQ1 with cell cycle inhibitors\textsuperscript{140}. The rationale behind this is discussed in more detail in the next section.

Targeting MYC through cell cycle inhibition

The cell cycle machinery is tightly regulated through several roadblocks making sure abnormal cells will not propagate. The uncontrolled proliferation of cancer cells tells us that they have acquired features helping them to escape the crucial checkpoints and slip through. Cells go through the four phases of the cell cycle; G1 (growth), S (DNA synthesis), G2 (growth), and M (mitosis) before actual cell division, cytokinesis at the very end of the M-phase (Fig. 4)\textsuperscript{114}. The process is controlled by regulatory proteins, cyclin-dependent kinases (CDKs), and cyclins all functioning at the different phases and checkpoints of the cell cycle. Cyclins and CDKs form heterodimers in which cyclins regulate the kinase activity of CDKs by binding. Different combinations of heterodimers function in the different phases of the cell cycle. The level of a particular cyclin fluctuates over the different phases and tunes progression when expressed and bound to its CDK partner\textsuperscript{114, 141, 142}. 
Figure 4. Extracellular growth signals lead to cell cycle progression by promoting transcription factor expression and activation. Subsequent expression of cell cycle genes such as cyclins pushes the cell through the phases of the cell cycle eventually leading to cell division. Inhibitory factors (dotted lines) are present at all stages and are the cell's security measures against e.g. DNA damage and other stressors that might lead to malignant proliferation.

An intricate array of extracellular growth stimulatory or inhibitory signals in G1 will lead to the expression of genes pushing cell cycle entry or quiescence (G0). The decision to progress is mandated by extracellular RAS/MAPK pathway signaling causing TFs such as MYC to be expressed. This will lead to a cell growth spurt, producing proteins and organelles to secure cell cycle progression. As a part of this, cyclins D1, D2, and D3 (collectively referred to as cyclin D) are produced and bind their partners CDK4 and CDK6. The active CDK4/6/cyclin D complexes hypophosphorylate the retinoblastoma protein (pRb), leading to increasingly unbound levels of the TF E2F otherwise bound to and inactivated by pRb\textsuperscript{143}. This is the start of a powerful feed-forward mechanism in which E2F promotes transcription of multiple late G1-phase and S-phase entry proteins, including cyclins E1 and E2 (collectively referred to as cyclin E). Cyclin E binds CDK2 that will hyperphosphorylate pRb, completely inactivating it and enabling E2Fs full transcriptional potential\textsuperscript{143, 144}. At this point, after the restriction point (RP), the cell is no longer dependent on extracellular signaling but runs according to a seemingly mitogenic independent preset cell cycle protocol\textsuperscript{114}. CDK2 not only plays a role in late G1- and S-phase entry but it is activated once again in the S-phase as cyclin E is replaced by cyclin A2 as the CDK2 partner. Cyclin A2 later binds CDK1 for
S-phase progression into G2. To enter the final cell cycle phase, mitosis, CDK1 forms a new complex with cyclin B (Fig. 4). There are several other cell cycle regulatory proteins besides the CDKs, cyclins, and regulatory proteins discussed above. Some of them have a more prominent role. p53 is a tumor suppressor important for DNA integrity control at the restriction point but also in G2/M. Activation of p53 due to DNA damage leads to the expression of regulatory proteins such as p21\(^{Cip1}\). p21\(^{Cip1}\) actively binds G1 CDK/cyclin complexes and blocks cell cycle progression long enough for the cell to repair the damage or if deemed irreparable the cell goes through p53 mediated apoptosis. Other CDK inhibitors include p27\(^{Kip1}\) (from here referred to as p27), p57\(^{Kip2}\), and INK4 proteins. INK4 proteins are specific CDK4/6 inhibitors (Fig. 4).

p27 is an important player in the feed-forward loop to get past the restriction point. p27 binds and inactivates CDK2/cyclin E through phosphorylation. This is counteracted by the increasing levels of CDK4/6/cyclin D complexes in the early G1-phase. CDK4/6/cyclin D is bound and even stimulated by p27, sequestering it from CDK2/cyclin E, allowing the first to hypophosphorylate pRb and the latter to hyperphosphorylated it. CDK2/cyclin E or A2 on the other hand phosphorylates p27 on threonine 187 (T187) leading to p27 degradation via the SCF\(^{SKP2}\) ubiquitin ligase complex (Fig. 5). In addition, it was recently shown that CDK1/cyclin A2 or B1 can also perform this function, replacing CDK2 if lost.

Figure 5. The integrative biology of MYC, CDK2, CDK4/6, and p27\(^{Kip1}\) in which p27\(^{Kip1}\) is involved in cell cycle progression.

MYC protein involvement and importance in cell proliferation, specifically cell cycle regulation, is widely studied and known. MYC promotes cell cycle progression...
progression in multiple ways, both directly by binding to the promoters of genes encoding CDKs and cyclins leading to transcription and indirectly by pushing mechanisms counteracting the anti-proliferative regulation of the cell cycle (reviewed in[148]) (Fig. 5). An example of the latter is MYC’s negative regulation of p27 by inducing expression of SKP2[149] and also cyclins D[150] and E[151].

Cell cycle perturbations are common in cancer. Key proteins are often mutated causing uncontrolled cell cycle progression and accumulation of DNA damage[152]. Inhibiting cell cycle proteins such as CDKs have shown promise in clinical trials. Targeted drugs towards specific CDKs are getting increased attention and seem to make better sense to use than pan CDK inhibitors that would cause more unwanted and off-target effects.

The FDA-approved drug palbociclib is a specific CDK4/6 inhibitor that causes G1 cell cycle arrest[153-157]. It has been tested in hormone receptor-positive, HER2-negative metastatic breast cancer with great success[158, 159], but also preclinically in neuroblastoma[160, 161]. Also, palbociclib was tested on MYCN-driven MB in paper I of this thesis[140]. In paper III, we tried a different but BBB penetrant CDK4/6 inhibitor[162], abemaciclib, which is in clinical trials for both adult and pediatric high grade and metastatic brain tumors (NCT02644460, NCT03220646).

CDK2 inhibition is of special interest to us due to its kinase targets and indirect dependence on MYC. CDK2 and MYC are involved in an intricate positive feedback loop in which MYC stimulates CDK2 activity by regulating the expression of cyclin A and E. And, active CDK2 stabilizes MYC by phosphorylating S62[116, 163] (Fig. 3). In addition, CDK2/cyclin E is important for cell cycle progression by hyperphosphorylation of pRb, as discussed above. Targeted inhibition of CDK2 is possible using e.g. milciclib, which is a CDK2 inhibitor that blocks entry past the restriction point[164]. Milciclib was used in papers I and III to target CDK2 in MYC-driven MB. Most CDK inhibitors are not specific but have an affinity for multiple CDKs and other kinases. It is therefore hard to evaluate the importance of specific CDK2 inhibition in MYC-driven cancer. To address this, we tried a potentially more selective CDK2 inhibitor, Nu6300[165] as well as knock-out of the Cdk2 gene in a mouse model of MYC-driven MB in paper III.
Present Investigations

Paper I. Combined BET bromodomain and CDK2 inhibition in MYC-driven medulloblastoma

Figure 6. BET bromodomain and CDK2 inhibition combined causes apoptosis and downregulation of MYC target genes in both a Group 3 MB mouse model and a MYC amplified MB xenograft.

MYC-driven medulloblastoma is correlated with poor survival. MYC proteins are TFs, known to be poor targets for therapy. However, alternative routes to target MYC have emerged as epigenetic targeted drugs have entered the arena. We have combined inhibition of BET bromodomains with specific inhibition of CDK2 using JQ1 and milciclib respectively to target MYC in medulloblastoma. Human and mouse MYC/MYCN dependent medulloblastoma cells were all sensitive to treatment with JQ1 and milciclib. Both as single agents
and in combination. Long-term combination treatment successfully abolished all tumor cells minimizing the risk of tumor recurrence. The combination of JQ1 and milciclib worked synergistically and halted the cell cycle, suppressed and destabilized MYC/MYCN, and further drove p53 mediated apoptosis. Moreover, the single agents sensitized medulloblastoma cells to radiation, which is commonly used to treat patients. Milciclib was shown to exert its effects mainly through CDK2 inhibition and not by off-target inhibition. Immediate transcriptional changes were identified using BET bromodomain inhibition and CDK2 blockage. Combining mouse and human RNA data demonstrated a clear benefit of using the combination treatment to target MYC/MYCN output. Lastly, the combination of JQ1 and milciclib increased the survival for allografted as well as xenografted MYCN/MYC-driven medulloblastoma-bearing mice.
Paper II. Dormant SOX9-positive cells behind MYC-driven medulloblastoma recurrence

**Figure 7.** A mouse model of MYCN-driven Group 3 MB recurrence. In a combined TetON/TetOFF system, MYCN is expressed from the Glt1 promoter to generate primary Group 3 MB. During a Tet-switch, enabled by doxycycline administration, MYCN expression is turned off from the Glt1 promoter and its expression is instead driven from the SOX9 promoter in SOX9 expressing quiescent cells. Primary tumors are cured and after approx. 100 days post-treatment the GTS model presents with metastatic recurrent tumors. Relapsed tumor cells are sensitive to MGMT inhibition as well as doxorubicin treatment.

Recurrent medulloblastoma is almost always fatal. Patients present with local recurrences or distant metastatic recurrences depending on the MB subgroup. The mechanisms behind MB recurrences are poorly understood and there are few in vivo models for studying relapse mechanisms at the molecular level. In the current paper, we study medulloblastoma recurrence in a novel mouse model based on the Group 3 MB model GTML. This model allows us to study the interactions of TFs SOX9 and MYCN in tumor recurrence. Also, new targeted therapies are being investigated to manage treatment-resistant relapsed MB.
In the previously published MYCN-driven GTML model of Group 3 MB, we identified a small population of non-dividing cells expressing the TF SOX9. These cells were distinct from the majority of tumor cells expressing MYCN and overlapped with a quiescent gene signature. The SOX9 positive cells were enriched upon MYCN depletion through dox administration and with cisplatin treatment. This would indicate that a quiescent SOX9 positive tumor cell population survives and is enriched when faced with standard treatment targeting fast-dividing cells.

SOX9 is correlated with poor survival in Group 3 MB and published single-cell genomics of Group 3 MB patients revealed a similar pattern of SOX9 expression to what had been identified in the GTML model. The majority of tumor cells had MYC expression, a small population was SOX9 positive and a third population expressing both TFs. The cells expressing only SOX9 displayed a quiescence signature. Interestingly, Group 3 and 4 patient data (primary-relapsed paired samples) further showed that the number of SOX9 positive cells was higher in the recurrent tumors compared to primary and that this could also be seen in higher metastatic grade Group 3 MB.

Based on these findings we developed a model of brain tumor relapse from the GTML model in which SOX9 plays a pivotal role. In the GTML model, MYCN is expressed from the Glt1 promoter and its expression can be turned off using dox in a Tet-OFF system that cures all GTML mice. We combined the GTML Tet-OFF model with a Tet-ON system in what we call the GTS model. In the GTS model, the dox treatment constitutes a Tet-switch so that MYCN expression is turned on from the SOX9 promoter. Similar to GTML, primary tumors occurred in the hindbrain and could be cured by dox treatment. But, in GTS, all mice relapse within 80-120 days. The recurrent GTS tumors were most often re-located to the forebrain and displayed SOX9 positive spinal cord metastases. Based on expression profiling and subsequent cross-species analysis the GTS tumors were identified as Group 3 MBs. In GTS, the SOX9 positive cells were also more quiescent and mostly mutually exclusive from MYCN expressing cells. Recurrence in the GTS system could also be studied in vitro by culturing cells from the primary mouse tumor (pre-GTS cells) and treat them with dox. Pre-GTS cells recovered and relapsed while GTML cells did not. Knock down of SOX9 using CRISPR-Cas9 in pre-GTS cells and dox treatment of the same proved that SOX9 was essential for tumor cell recovery.

Lastly, we identified MGMT as a potential target for recurrence-specific treatment. And, by comparing the enrichment scores of forebrain GTS and hindbrain GTS cells against the chemical and genetic perturbation (CGP) database of gene sets we could identify doxorubicin as a recurrence-specific drug.
Figure 8. *Cdk2* is knocked out *in vitro* in a novel MYC-driven Group 3 MB model (GMYC) using tamoxifen-induced Cre recombination. CDK2 depleted cells exhibit high levels of p27<sup>Kip1</sup>, proliferate slower than CDK2 wildtype cells, and upregulate genes involved in mTOR signaling as well as ZMYM2 targets genes. Lastly, *Cdk2* knock-out in tumor cells prolongs the survival of allografted mice.  

In paper III we wanted to continue exploring CDK2 and MYC for targeting Group 3 MB. In paper I we mainly used the mouse model GTML, which is an MYCN-driven model<sup>57, 140</sup>. MYCN overexpression in Group 3 MB is rather rare<sup>17</sup> and instead, we decided to use the newly developed (in our group) and unpublished MYC-driven GMYC model that recapitulates human Group 3 MB, specifically the subtype 3<sup>g63</sup>. We bred the GMYC model with an R26CreERT2; *Cdk2*<sup>fl/fl</sup> model to be able to study the optimal targeted CDK2 treatment, knock-out of *Cdk2*, using tamoxifen-induced Cre. The main part of the study was conducted on cultured tumor cells from the aforementioned tumor model, with wild-type *Cdk2* or *Cdk2* knock-out (GEC-WT and GEC-KO respectively).  

Concurring with previous data published by us and others<sup>140, 166-168</sup>, specific CDK2 inhibition (hereby *Cdk2* knock-out) did not kill the tumor cells but merely slowed their proliferation rate. And, we found no compensation by other CDKs that we could target pharmacologically. In this model, CDK2 seemed not to be important for MYC activation as seen by unchanged protein levels of pS62 MYC. However, the protein levels of the endogenous CDK2
inhibitor p27\textsuperscript{Kip1} were higher in Cdk2 knock-out cells compared to wild-type. MYC promotes expression of the CDK2 binding partner cyclin E, which in complex with CDK2 is phosphorylated by p27\textsuperscript{Kip1} and vice versa for Skp2 mediated degradation. Based on this, we propose a mechanism in which CDK2 inhibition in MYC driven Group 3 MB leads to p27\textsuperscript{Kip1} dependent cell cycle arrest, but not cell death. Among the top differentially expressed gene sets in knock-out cells as compared to wildtype, ZMYM2\_TARGET\_GENES were significantly upregulated in GEC-KO cells. ZMYM2 is connected to CDK2 through the transcription factor B-Myb, known to drive proliferation and cell survival. We propose to indirectly inhibit the proliferative capacity of B-Myb in combination with CDK2 depletion by targeting mediators of B-Myb activity such as Aurora kinase B and Survivin.
Conclusions

This thesis has addressed targeted therapies and recurrence mechanisms of medulloblastoma. We present novel therapeutic strategies to treat MYC-driven medulloblastoma and dive deeper into the role of cell cycle inhibition in this type of cancer. Furthermore, we report the first mouse model of relapsed medulloblastoma and present a novel mediator of the same. The thesis includes three papers, conclusions of each are summarized below.

Paper I
We presented a combination treatment of MYC/MYCN-driven medulloblastoma consisting of CDK2 and BET bromodomain inhibition. This combination caused apoptotic cell death and prolonged survival of mice with MYC amplified medulloblastoma.

Paper II
For the first time, medulloblastoma relapse could be modeled in an immunocompetent spontaneous mouse model. We propose a mechanism by which SOX9 is needed to promote recurrence in MYC-driven medulloblastoma. Quiescent SOX9-positive cells were enriched upon treatment and were later reactivated to form distant metastatic recurrences. These recurrences could be treated with MGMT inhibition and doxorubicin.

Paper III
We further investigated targeted therapy of MYC-driven medulloblastoma using a novel mouse model to improve the treatment presented in paper I. Conditional knock-out of Cdk2 was used to study CDK2 depletion in tumor cells which proved to have minor effects on global expression levels and no effect on MYC stability or activity. Cdk2 depletion led to a decreased proliferation rate of the tumor cells. Suggested to be explained by p27Kip1 mediated cell cycle arrest. Moreover, as identified from expression analysis, we proposed to target mediators of B-myb transcriptional activity in combination with CDK2 inhibition as a means of treating MYC-driven medulloblastoma.
Discussion and Future Perspectives

New sequencing technologies have produced enormous amounts of data on medulloblastomas. Paper after paper is suggesting new targets for therapy to successfully treat both primary and recurrent tumors by either developing new inhibitors or use already existing ones for new indications. Including the papers of this thesis. In addition to this, the hard work and ingenuity of the research community has resulted in novel ways of treating highly aggressive MYC-driven MB tumors that do not only involve the classic small molecule inhibitors (reviewed in\textsuperscript{127}) such as immunotherapies\textsuperscript{169, 170} and tumor treating fields\textsuperscript{171}. As this is still in its early days and knowing that good and sound science often takes time, we can only hope that this will lead to approved and working treatment options that will benefit MB patients. Current treatment is successful for some MB subgroups but for those with high-risk disease such as those that are young, treatment-refractory, or later relapse much is yet to be done. One of the biggest challenges that this field is faced with is to significantly improve or even find a replacement for radiotherapy. Irradiation is responsible for most of the life-long side effects of MB treatment\textsuperscript{40} and on the other hand it is the most successful treatment, together with surgery, of this highly aggressive tumor\textsuperscript{41, 42}. This is highlighted by the dismal prognosis of young children and infants that are not eligible for radiotherapy\textsuperscript{43}. Many different radiotherapy technologies exist, some have the potential to decrease the damage to healthy tissue (reviewed in\textsuperscript{40}). Despite promising data, more time is needed to evaluate both the safety and efficacy of those. Another means of improving the outcome of radiotherapy is finding radiosensitizers that could be combined with irradiation to perhaps lower the dose. My thesis is mainly focused on tumor modeling and using that as a means of studying new targets of treatment as well as mechanisms of MB relapse. I hope that this work will contribute to a better future for children diagnosed with MB.

By using our expertise in MYC biology we have managed to successfully treat MYC-driven MB models by combining BET bromodomain inhibitors and CDK2 inhibitors in paper I\textsuperscript{140}. Prolonged survival of both the MYCN-driven GTML model as well as an MYC amplified human MB xenograft was the result of this treatment combination\textsuperscript{140}.

We wanted to do more and asked if there were better targets to combine with CDK2 inhibition to decrease the burden on normal cells and at the same time improve efficacy on tumor cells.
Paper III is in many aspects a continuation of paper I. Knock-out of Cdk2 is the optimal CDK2 inhibition, something that we did in a new mouse model of Group 3 MB, GMYC. This was done in vitro and allowed us to investigate how CDK2 depletion could be compensated in this MB model. Cdk2 knock-out on its own had very little effect on the tumor cells, as expected\textsuperscript{140, 166-168}. But, to our surprise, it also had minor consequences on global gene expression. In addition, CDK2 was not important for MYC activity in contrast to what others have reported and we showed in paper I\textsuperscript{140, 172, 173}. Of note, the models used in these two papers are different. Still, we have of yet an explanation for this.

In the continuation of paper III we will take advantage of the sparse information we did get from the expression analysis to see if there are means of targeting CDK2 together with mediators of B-myb activity and/or mTOR pathway signaling. Moreover, we will explore the mechanism behind Cdk2 knock-out mediated lowered proliferation rate by checking for senescence, apoptosis and by doing cell cycle analysis. CDK1 mediated compensation for knockdown of the other interphase CDKs is a known phenomenon explained by CDK1’s ability to bind all types of cyclins\textsuperscript{167}. Despite the lack of evidence for such mechanisms occurring in our model this still has to be explored on protein level with e.g. co-immunoprecipitation. Cdk2 knock-out allografted mice had prolonged survival compared to Cdk2 wildtype. Possibly explained by the slower proliferation. Similar to what we published in paper I, targeting CDK2 alone will not prolong survival substantially, and we will try any promising combination treatments found from in vitro testing also in allografted mice. In addition, with our GMYC model, we have a unique opportunity to test if CDK2 can function as a radiosensitizer in highly aggressive MB. What is still unknown is Cdk2’s role in tumor initiation. To answer that question we will knock out Cdk2 postnatally in GMYC mice, a protocol that is under optimization. The questions remain, how can these tumor cells divide without CDK2 and no compensatory action by other CDKs? And, is CDK2 inhibition worth pursuing in patients?

The transcription factor SOX9 is in the spotlight of paper II where SOX9 expression is needed for relapse to occur in the GTS model. This paper aimed to present and evaluate the first model of MB relapse. Despite the increasing understanding of MB recurrence we have yet been able to use this information to decrease neither metastasis nor relapse frequencies. This paper is an important contribution towards that end. SOX9 positive tumor cells represent a small population of, probably, quiescent cells that can be enriched upon treatment and later be reactivated to give rise to a distant recurrent tumor. In other words, we propose that the tumor bulk consists of plastic cell states that can shift from highly aggressive and proliferative to migratory, non-proliferative cells and back. The exact mechanism of how this occurs is not uncovered in the paper. However, this notion is further based on the anti-correlating
relationship between MYCN (proliferation) and SOX9 (quiescence) in the GTS model as well as the fact that both of these can be expressed in the same cell on the expense of expression of the other. No direct regulation between these two TFs could be discerned. The mechanism behind this could therefore simply be a result of large molecular programs regulating cell states. This should be addressed in future studies to reveal means of targeting the risk of recurrence already at diagnosis. One effort of fate-mapping tumor cells in our model is the GTML;pSOX9;GFP in which we can follow SOX9 expressing cells in the GTML model with GFP. Preferably, a similar approach should also be used in GTS to fully uncover the underlying events at the “Tet switch” point, when MYCN is driven from the SOX9 promoter and later during migration and relapse.

The GTS model offers the only means of studying spontaneous primary and relapsed MB tumors in the mouse and is a good complement to the recently increased number of publications of recurrent MB. Patient data, either paired primary-recurrent or unpaired offers snapshots at tumor presentation for us to understand how tumors have changed after treatment. The GTS model however could be used to study means of dissemination to further understand how and when some tumor cells can migrate to give rise to metastases or local recurrences. With the addition of standard therapy, one might also be able to understand the role of treatment for tumor relapse. The benefit of using a model with an intact immune system is that we can study the role of the tumor microenvironment including immune cells and inflammatory factors that might contribute to metastatic spread and treatment resistance. We reported that recurrent tumors in our model indeed are more inflammatory and express markers of immune escape. Finally, the TetON/TetOFF combined system is currently being incorporated into the newer GMYC model that could be used for future studies.

All in all, this book was written with every medulloblastoma patient and their family at heart. I truly hope that my and our work will be read, scrutinized, and used by the research community for the benefit of knowledge and in the end, for those that desperately need it to survive their cancer.
Thesis summary in Swedish


Avhandlingen är en sammanfattning av tre studier gjorda på medulloblastom. Dessa sammanfattas kortfattat enligt nedan:

I Genom att använda kombinationsbehandling för att slå mot epigenetisk reglering och cellcykelproteiner kunde vi öka överlevnaden hos medulloblastom med höga nivåer av MYC i djurmodeller.

II Vi identifierade transkriptionsfaktorn SOX9 som en avgörande faktor för återfall i den första musmodellen av medulloblastom-recidiv.

III Genom att, med genetiska metoder, ta bort cellcykelproteinet CDK2 i ny musmodell kunde vi dra slutsatsen att enbart förlorat CDK2 inte påverkar medulloblastomceller med högt MYC i någon större utsträckning. Vi hoppas att identifiera hur det ändå kan känsliggöra tumörceller för annan riktad behandling.
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References


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