Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Dag Hammarskjölds väg 20, Uppsala, Friday, 11 March 2016 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Assistant Professor Ulrich Schüller (Ludwig-Maximilians-Universität München, Germany).

Abstract


Medulloblastoma (MB) is the most common malignant pediatric brain tumor. Overall survival is about 70% and in cases where current treatment fails, the disease recurs and most often is fatal. At the molecular level, MB can be divided into four defined subgroups: WNT, SHH, Group 3 and Group 4. Amplification of MYC family genes is common in MB and correlates with poor prognosis and tumor relapse.

In this thesis we showed how MYCN initiates brain tumors when transduced in neural stem cells (NSCs). Prior to transduction, NSCs were isolated from different brain regions and at various time points. While overexpression of wild-type MYCN did not generate any tumors, orthotopic transplantation of MYCN<sup>T58A</sup>-expressing forebrain, brain stem and cerebellar NSCs induced diffuse malignant glioma, PNET-like tumors and MB, respectively. Interestingly, MYCN<sup>T58A</sup>-expressing cerebellar NSCs induced SHH-dependent MB from embryonic cells but SHH-independent MB from postnatal cells. We further showed that cerebellar NSCs transduced with both MYCN<sup>T58A</sup> and transcription factor SOX9 developed tumors faster and promoted distant migration into the forebrain.

The function and regulation of SOX9 in MB cells is poorly understood. We identified SOX9 protein as target of FBW7 ubiquitin ligase and demonstrated the effects of SOX9 on MB cells migration, metastasis and drug resistance. We further blocked PI3K pathway to destabilize SOX9 which sensitized cells to cytostatic treatment.

We used a (TetOFF) transgenic mouse model of MYCN-induced MB (GTML) and crossed it with a (TetON) transgene which allowed us to specifically target rare SOX9-positive cells in the tumor. In this system, MB develops spontaneously and SOX9-negative tumor cells can be killed off by doxycycline. The few remaining SOX9-positive cancer cells were able to promote distant MB recurrences. Such a pattern of relapse was recently shown for Group 3 and 4 human MB where about 90% of the recurrences were distant.

In summary, this thesis demonstrates that MYCN can generate various types of brain tumors depending on the timing and location of its expression. It further defines the existence of a rare population of SOX9-expressing MB cells that are involved in causing distant MB recurrences. Finally, it describes how SOX9 is stabilized in MB cells and increases MB migration and therapy resistance.

Keywords: Medulloblastoma, SOX9, MYCN, cancer development, recurrence, regulation, tumor metastasis, migration

Vasil Savov, Department of Immunology, Genetics and Pathology, Rudbecklaboratoriet, Uppsala University, SE-751 85 Uppsala, Sweden.

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ISSN 1651-6206
urn:nbn:se:uu:diva-274630 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-274630)
Времето е в нас и ние сме във времето.
То нас обръща и ние него обръщаме.

- Васил Левски (1871)

Time is within us and we are within time.
It changes us and we change it.

- Vasil Levski (1871)
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


*, **, # These authors contributed equally to this work

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Abbreviations

ASLV  Avian Sarcoma Leucosis Virus  
BCC   Basal Cell Carcinoma  
CD    Campomelic Dysplasia  
CNS   Central Nervous System  
CRE   Causes Recombination  
CSF   Cerebrospinal Fluid  
DF-1  Immortalized Chicken Derived Fibroblast cell line  
Dox   Doxycycline  
DUB   Deubiquitinating enzyme  
ECM   Extracellular Matrix  
EMT   Epithelial-Mesenchymal Transition  
GFAP  Glial Fibrillary Acidic Protein  
HSV   Herpes Simplex Virus  
LRL   Lower Rhombic Lip  
MB    Medulloblastoma  
NSC   Neural Stem Cells  
PDGFRα Platelet-Derived Growth Factor Receptor alpha  
PNET  Primitive Neuroectodermal Tumor  
PRS   Pierre Robin Syndrome  
PTCH  Patched  
RCAS  Replication-Competent ASLV LRT with a Splice acceptor  
rT TA Reverse Tetracycline-controllable TransActivator  
SHH   Sonic Hedgehog  
SMO   Smoothed  
SOX9  Sex-determining Region Y (SRY) box 9  
SRY   Sex-determining Region Y  
SUFU  Suppressor of Fused homolog  
SVZ   Subventricular Zone  
Tet   Tetracycline  
TetO  Tet Operator element  
TMA   Tissue Microarray  
tT TA Tetracycline-controllable Transactivator  
Ub    Ubiquitin  
UPS   Ubiquitin Proteasome System  
WHO   World Health Organization  
WNT  Wingless
Introduction

Medulloblastoma

Cancer is among the leading causes of childhood mortality. Leukemias are the most prevalent cancer type in children, followed by brain tumors, lymphomas and neuroblastoma. Brain tumors are further divided according to World Health Organization (WHO) - established criteria, into different diseases based on their localization, clinical features, histopathology, immuno-reactivity and genetics. This classification is used to predict tumor behavior and outcome, and neoplasms are categorized as grade I to IV with grade IV being the most aggressive and having the worst prognosis. The most common pediatric malignant brain tumor is medulloblastoma. It is classified as WHO grade IV and affects approximately six out of one million children. Medulloblastomas develop in or around the cerebellum and show a tendency to metastasize via the cerebrospinal fluid (CSF) to the forebrain and spine. More boys are affected than girls with 65% of diagnoses occurring in males. The survival rate is currently 70-80% however survivors experience long-term side effects due to the aggressive nature of disease treatments.

Medulloblastoma subgroups

Histopathological analysis currently classifies medulloblastoma into five groups including (a) classic, (b) desmoplastic/nodular, (c) medulloblastoma with extensive nodularity, (d) large cell medulloblastoma, and (e) anaplastic medulloblastoma. Medulloblastoma has also been divided into various subgroups according to the gene expression profiles of tumor samples (Figure 1). During the past ten years, research groups worldwide have clustered the samples into 4 to 6 subclasses. Recently, a consensus nomenclature was created with four molecular subgroups: WNT (Wingless), SHH (Sonic Hedgehog), Group 3 and Group 4. WNT and SHH subgroups are named after the developmental pathway which was found to be over activated in these tumors. Approximately 5-10% of the malignancies are classified into the WNT subgroup and around 30% into SHH. WNT patients have the highest survival rate greater than 90% while the SHH subgroup shows 70% survival.

It is less known what signaling pathways or acquired mutations are involved in or drive the other two subgroups. Therefore, these groups are...
simply called Group 3 and Group 4. Together they account for more than 60% of medulloblastoma cases and the survival of Group 3 patients is the poorest (around 50%).

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>WNT (~10%)</th>
<th>SHH (~30%)</th>
<th>Group 3 (~25%)</th>
<th>Group 4 (~35%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender ratio (M/F)</td>
<td>1/1</td>
<td>1.5/1</td>
<td>2/1</td>
<td>3/1</td>
</tr>
<tr>
<td>Age distribution</td>
<td>Infant, Childhood, Adult</td>
<td>Infant, Childhood, Adult</td>
<td>Infant, Childhood, Adult</td>
<td>Infant, Childhood, Adult</td>
</tr>
<tr>
<td>Histology</td>
<td>Classic; very rare, LCA</td>
<td>Classic; &gt;dysplastic/nodular &gt; LCA &gt; MIBEN</td>
<td>Classic; &gt;LCA</td>
<td>Classic; rarely, LCA</td>
</tr>
<tr>
<td>Metastasis at diagnosis</td>
<td>~5-10%</td>
<td>15-20%</td>
<td>~40-45%</td>
<td>~35-40%</td>
</tr>
<tr>
<td>Overall survival (5 years)</td>
<td>~95%</td>
<td>~75%</td>
<td>~50%</td>
<td>~75%</td>
</tr>
<tr>
<td>Proposed cell of origin</td>
<td>Lower rhombic lip progenitor cells</td>
<td>CGN + % of the EGI and cochlear nucleus; neural stem cells of the SVZ</td>
<td>Prominin 1, lineage; neural stem cells; CGN + % of the EGI</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

**Figure 1.** Molecular classification of medulloblastoma into four molecular subgroups. Along with different molecular profile, the four groups show diverse histology, genetics, metastasis and survival rates (modified from).

**MYC family in medulloblastoma**

The MYC family of transcription factors includes three members; MYC (or C-MYC), MYCN and MYCL. They have important roles in various cancer types. Their functions and target genes have been thoroughly investigated. However, studies about MYC proteins present different suggestions about the biological role of MYC proteins which are often not related to their oncogenicity. That being said, a role as a universal amplifier of transcription has been proposed. Two research groups demonstrated how MYC can increase the overall levels of transcription in cells. These results were generated in tumor cells, embryonic stem cells and in differentiated lymphocytes which suggests that the process is universal.

Amplifications of all of the MYC family genes have been described in medulloblastoma and amplifications of MYC or MYCN correlate with poor survival, large cell/anaplastic (LC/A) phenotype and higher metastasis rate. However, although elevated expression levels of MYC proteins are often found in tumors, they do not correlate with high-risk disease and cannot be used as a prognostic marker.

The role of MYC transcription factors in medulloblastoma can vary, depending on the molecular subgroup of the tumor. Increased expression of all of the MYC genes is found in all MB subgroups but these elevated levels are thought to sometimes result from active upstream signaling in the tu-
Mors. For example, in the WNT subgroup all of the MYC genes have high expression but none of them have been found to be amplified. Most patients with WNT tumors have a very good prognosis and this does not seem to be affected by the high MYC levels. High MYC expression may be a result of the increased WNT pathway activity as it has been shown to be a target of upstream WNT signaling. The situation may be similar in the SHH group where MYCN is induced by SHH signaling. However, amplifications of MYCL and MYCN were specifically found in this group and MYCN amplifications were associated with poor outcome. There are mouse models showing that SHH tumors may be induced by increasing MYCN expression. Amplifications of MYC occur only in the most aggressive, Group 3 tumors. There are also rare MYCN amplifications found in this subgroup. The expression of MYC is high compared to the SHH group and Group 4 but is similar to WNT, and again, it is the amplifications which may be used as predictive factor. There are amplifications of MYCN also in Group 4. MYCN is able to induce Group 3-like tumors when overexpressed in transgenic or viral mouse models.

MYC proteins are very unstable and their half-life is in the range of 20-30 minutes. Suppression of the turnover of MYC family proteins is involved in tumor development. The viral proto-oncogene v-Myc has a Thr58Ala mutation that was first reported in Burkitt’s lymphoma patients. Most of the viral transcripts used for modeling the medulloblastoma have mutation at this position. This residue is where MYC proteins become phosphorylated by GSK3β in order to be marked for degradation by the ubiquitin-proteasome system. The ubiquitin ligase which recognizes this phosphorylated site and initiates the ubiquitylation is FBW7. Its expression is downregulated in medulloblastoma and other brain tumors.

Treatment

Medulloblastoma treatment includes surgery, radiotherapy and chemotherapy. The intensiveness of the treatment varies and is dependent on the age of the patient and the progression of the disease. The amount or dose of radiation used in photon radiation therapy is measured in gray (Gy). Usually, the radiation therapy is directed against the tumor bed and the dose is between 54-55.8 Gy. Children under four years of age are usually not irradiated. When necessary, whole cranial and spinal irradiation in children over three years of age is performed at a lower dose (23-35 Gy). The chemotherapy which follows depends on the particular case and is decided individually.

The current treatment strategies are often effective and many children survive the disease (survival rate 75-80%). However, the treatment regimens are aggressive and patients are often very young, with underdeveloped nervous systems. Hence the vast majority of patients, although free of disease, develop long-term side effects which can seriously affect their quality
of life. Adverse effects from therapy in young patients often include neurological defects, a lower IQ and cognitive impairment.

Recurrence

If tumor cells survive treatment, they have developed a resistance and can regenerate the tumor or promote tumor progression. This process is called tumor recurrence. In many tumor types, it co-occurs with metastasis, the process of tumor spread to other organs outside of the initial tumor site. Metastasis has been studied intensely in many cancers and particular signaling pathways and molecules have been found to take part at different steps in this process, although the mechanisms are still not fully understood.

An unfavorable outcome in medulloblastoma and brain tumors is almost always due to tumor relapse and disease progression. In general, CNS cancers very rarely metastasize outside the nervous system. A recent observation suggests that embryonal malignancies tend to either recur locally, where the initial tumor occurred, or via leptomeningeal spread. This did not appear to be the case with an embryonal tumor medulloblastoma which also formed distant relapses in the forebrain. Another study followed the biological nature of medulloblastoma recurrence and discovered that these tumors always match the molecular signature of the initial tumor subgroup. While WNT group recurrences are very rare, all other subgroups can relapse. Survival after such recurrence is extremely poor (approx. 10% 5-year survival) with a median survival of less than 2 years. The site of tumor relapse is not always at the position where the primary medulloblastoma was located. Generally, SHH medulloblastomas seem to recur at the same position where the initial tumor develops (tumor bed) whereas SHH independent tumors most often include distant (metastatic) growth(s). These findings point out yet again the importance of subgrouping these tumors and the possibility to use subgrouping to predict the nature of possible relapses. It is also highly likely that depending on the classification, there are different mechanisms for tumor spreading and recurrence. Further investigations in this direction may lead to the development of treatment preventing relapse, thereby improving the dismal outcome of recurrent medulloblastoma.
Mouse models in cancer

Animal models are an important tool for studying cancer development. Modeling the disease *in vivo* is the only way to study all the interactions and pathologies which appear in a complex living organism. Animals are also vital for finding the factors involved in the initiation and progression of the disease *in vivo* and the only way to verify findings which were made in laboratory settings (*in vitro*).

Mice are the most commonly used laboratory animals. Their short breeding time, small size, high similarity to humans, large offspring and short lifetime make them extremely valuable to scientific research. Two common ways to use mice in experiments are to generate a transgenic mouse or to manipulate cells *in vitro* and later transplant them back into the animal (*ex vivo*).

Transgenic animals can be modified to completely lack one or several genes (knock-out) or to express more copies of one or several genes or genes altered in structure (knock-in)\(^48\). Although this is a useful method to study gene function, complete knock-outs and knock-ins have some disadvantages. One of these is that the gene of interest is expressed (or deleted) in all the tissues and organs of the animal or that the gene is lethal in embryonic development. Whenever problematic, this can be bypassed by using a tissue-specific promotor to temporally and/or spatially regulate the expression. It is, however, not trivial to find suitable cell-specific promoters. To overcome embryonal lethality, regulatable systems are also used. In this way the deletion or overexpression of the gene of interest is induced at a desirable stage in the development to avoid complications in the embryonic phase\(^49\). Tetacycline regulatable systems and tamoxifen-induced CRE recombination are the most widely used regulatable systems. In this thesis, I describe in detail the tet system which was used in our studies.

The function of a gene may also be studied after its introduction to a particular cell population and the consecutive injection of these cells into animals. Another experimental approach is to inject viral particles *in vivo* or electroporate foreign DNA (often *in utero*)\(^48\). These methods are faster than engineering a transgenic mouse but always include laboratory interventions which remove the cells from an intact living organism and introduce them to an artificial environment. Specificity in targeting particular cell populations may be achieved using the RCAS/tv-a model which is described later, or by using a tissue-specific promotor in the constructs. The tet or CRE regulatable systems may be integrated in different constructs and the function of the gene of interest may be also studied at desired developmental time points.
Figure 2. Mechanism of “tet on” and “tet off” regulatable systems. tTA or rtTA are induced in cells usually by tissue specific promotor (TSP). In order to activate transcription of desired transgene (Tg) from tetO promoter, rtTA needs to bind doxycycline (“tet on” system). The reverse situation is true for tTA. It is constitutively active but its activity may be inhibited by doxycycline (“tet off” system).

Tet regulatable transgenic models

Tetracycline (tet) or its derivative doxycycline (dox) are used in the so-called tetON and tetOFF systems to achieve a regulatable expression of a desired gene (Figure 2). The method has been developed by Bujard⁵⁰ and is based on a fusion protein, tTA. This is a combination of the tetracycline repressor (tetR) Tn10 transposon element of *E. coli* with the transactivation domain of the Herpes Simplex Virus (HSV) virion protein 16. The resulting tetracycline-controllable transactivator (tTA) is able to activate a transcription promoter called the tet operator (tetO) element⁵⁰. The transcription activation is blocked by doxycycline. Another transactivator called reverse tTA (rtTA) acts in the opposite way and activates transcription only in the presence of dox⁵¹. The system using tTA is known as tetOFF because it is blocked by tet or dox and the rtTA based system is known as tetON because it is activated by tet or dox.

In transgenic mouse engineering, these two systems are widely used. The specificity of the models is achieved by driving the tTA or rtTA from a tis-
sue specific promoter. In this way the transactivator molecules will be expressed only in this particular cell type\textsuperscript{51}. The gene(s) whose expression is controlled is situated on a separate transgene under the control of tetO promoter. By administration of dox, the expression of this gene(s) is turned on (if rtTA) or turned off (if tTA)\textsuperscript{49}.

Figure 3. Use of RCAS/Tv-a system to follow brain tumor development from GFAP-positive NSCs. RCAS viruses are amplified in chicken DF1 cells. Supernatant with viral particles is used as culture media for cells isolated from transgenic mice (expressing avian TVA receptors from cell specific promoter). In this way the gene of interest is delivered only to NSCs that express TVA virus receptor. (modified from

RCAS/Tv-a system

An elegant tool to deliver DNA to particular brain cell types has been developed by H. Varmus and E. Holland\textsuperscript{52,53}. The system is based on the use of specific transgenic mice which express receptors (TVA) for avian sarcoma leucosis virus (ASLV). Since these receptors are not normally expressed in mice, different transgenic mouse strains have been generated where TVA expression can be induced from different tissue specific promoters. A list of all published TVA transgenic and knock-in mouse strains is included in a recent protocol article\textsuperscript{54}.
Infection with Rous Sarcoma Virus (RSV) - derived Replication-Competent ASLV LRT with a Splice acceptor (RCAS) cloning vector delivers the DNA that has been inserted in it to the cells which express the TVA receptor. In order to amplify the virus in culture, chicken-derived fibroblast cells (DF1) are used. They are transduced with the RCAS vector and quickly start producing retroviral particles into the culture media, which is then collected. The virus-containing solution may be used to infect cells in vitro, or alternatively, DF1 cells may be injected in mouse and the virus produced by them infects the nearby cells. In species other than birds these retroviruses are replication-incompetent. This system provides high specificity which allows for the study of the gene’s effect on a particular cell type. However, the transfection efficiency is low and the virus infects only dividing cells.

Mouse models of medulloblastoma

Many animal models have been created to study medulloblastoma development in vivo. Most of them model SHH tumors but the other molecular subgroups have now also been recreated. Both viral vectors and transgene-based methods have been implemented.

WNT tumors were generated in a transgenic model by overexpressing a mutant ctnnb1 transcript in certain lower rhombic lip cells in the developing brain stem. The model resembles the human condition and suggests a brain stem origin rather than a cerebellar origin, as in other WNT tumors. In this MB model, tumors only develop when p53 is knocked out. However, in patients, mutations of p53 are seldom found in WNT tumors.

Multiple SHH subgroup models have been developed. The first was the PTCH knock-out mouse which mimics Gorlin syndrome caused by loss-of-function mutations in the PTCH gene. This mouse develops SHH MB if it is heterozygous for the mutant allele. Other models mimic mutation or aberrant expression of some of the other members of the SHH pathway like constitutive activation of SMO or SHH or SUFU (together with Trp53) deletion. The different models were induced in various cell types like cerebellar granule neuron precursors, GFAP (Glial Fibrillary Acidic Protein) or Nestin-expressing cells, as well as different systems (transgenic animals, RCAS, electroporation, etc.)59. There are also transposon-based systems which aim to identify genes involved in MB dissemination and metastasis.

Few SHH-independent models have been developed to date. Two of them are based on MYCN activation. In the first transgenic model a tetOFF system is used to generate increased expression of MYCN. This results in Group 3 or Group 4 tumors but rarely in SHH MB tumors, similarly to what is observed in patients. The second model is the MYCN-driven RCAS-based model. It targets GFAP-positive cells in the cerebellum which are infected with mutationally-stabilized MYCN. Depending on the developmental stage
of MYCN overexpression, either SHH or Group 4 tumors develop. The article presenting the RCAS viral model is part of the current thesis\textsuperscript{31}.

Two models of Group 3 tumors were developed for this aggressive anaplastic type of MB\textsuperscript{38,72}. They are both based on MYC overexpression by viral infection \textit{in vitro} and subsequent transplantation of the cells in mouse brain. Interestingly, both of the articles that discuss these models showed that a loss of p53 was required to induce MB, and this is not the case in human Group 3 MB\textsuperscript{61}.

Comparing the existing mouse models with human MB subgroups based on gene expression profiles and sequencing data, scientists observed that some of the Group 3 mouse models match close to the human SHH subgroup\textsuperscript{73}. According to their work, the best model for Group 3 MB turned out to be the GTML model although it uses MYCN\textsuperscript{33} as driving oncogene and MYCN amplification was found in only 2-3% of Group 3 MB patients\textsuperscript{27}.

Medulloblastoma mouse models have increased our knowledge about tumor initiation and development\textsuperscript{31,74}. However, more work is needed to understand how and why tumor recurrence develops. Recently, researchers performed a surgical and radiation procedure on a mouse model of SHH medulloblastoma in order to recreate patient treatment\textsuperscript{75}. They discovered that a minor population of cells in the primary tumor (<5%) is genetically identical with the recurrent tumor suggesting that a few calls are able to recreate the whole relapse.
SOX9

The Sex-determining Region Y (SRY) box 9 or SOX9 is a transcription factor that belongs to group E of the SOX family of proteins. There are approximately 20 SOX proteins in mammals and they are further characterized by their high mobility group (HMG) box domain. SOX9 is involved in the development of multiple organs but is also dysregulated in various cancers. Loss-of-function mutations in the gene or the surrounding DNA cause a syndrome called campomelic dysplasia (CD). It is characterized by problems in bone development and 46, XY sex reversal (failed testis development). The patients also show defects in kidney, heart, brain and pancreas development. Regions around the SOX9 gene have been associated with Pierre Robin syndrome (PRS), a genetic disorder with facial malformations.

SOX9 in normal development

SOX9 is involved in a number of developmental processes. Neural crest development is regulated by SOX9 which is expressed in early neural crest cells. These cells need to migrate to form different body parts of the embryo. However, in order to become migratory mesenchymal cells, they undergo epithelial to mesenchymal transition (EMT). It was shown that SOX9 cooperates with one of the main regulators of EMT, Slug, to promote its transcription and in this way to facilitate this process. Forced expression of SOX9 and Slug was able to induce EMT in chick neural tubes.

SOX9 expression is necessary in order to induce and maintain NSCs in mouse and chicken embryos. It was also found to be the main target of miR124 in a study describing adult neurogenesis in the SVZ (SubVentricular Zone). SOX9 determines glial cell fate and is expressed in glial populations of cells in the developing and developed brain. In spinal cord development, it acts together with SOX10 to induce PDGFRα which influences the survival and migration of oligodendrocyte precursors.

Chondrogenesis also requires SOX9 function as it is a regulator of the transcription of several ECM proteins required for this process. In sex determination, SOX9 is one of the main factors. It is induced by SRY together with SF1 and, if duplicated in humans, it alone may induce 46, XX female-to-male sex reversal. This means that SOX9 is essential for testis development which fails in CD. Involvement of SOX9 in the development of the prostate, pancreas and intestine has also been investigated. In these organs, SOX9 is expressed in tissue-specific progenitors but not in terminally differentiated cells.
SOX9 in cancer

SOX9 was found to be highly expressed or mutated in different types of cancer. It has never been shown to induce tumors alone but several articles demonstrate that it can increase tumor aggressiveness, proliferation and metastasis\textsuperscript{31,101-103}. SOX9 was described in a recent study to be involved, together with Slug, in inducing and maintaining the ability of breast cancer cells to metastasize\textsuperscript{104}. The authors also succeeded in blocking this ability in cells which have been shown to metastasize upon injection. SOX9 was also involved in the first steps of Kras-induced pancreatic cancer\textsuperscript{105}. If SOX9 overexpression is combined with PTEN loss in the prostate it can generate cancer\textsuperscript{106}. It also cooperates with NMYC in medulloblastoma\textsuperscript{31} and H-ras in colorectal cancer\textsuperscript{101}.

Mutations in SOX9 are rarely found in cancer but they may occur in colorectal cancer\textsuperscript{107}. Alternative splicing is another mechanism that might be related to SOX9 oncogenic functions\textsuperscript{108}. Elevated SOX9 expression has been detected in human cancers such as lung, medulloblastoma, pancreatic, ovarian, prostate, neuroglia, breast and thyroid\textsuperscript{101}. This expression was subtype-specific in medulloblastoma where SOX9 was expressed in WNT and SHH groups but not in Group 3 and Group 4\textsuperscript{31}. High SOX9 levels were also implicated in another SHH malignancy – basal cell carcinoma (BCC)\textsuperscript{109}. In skin cell development SOX9 is a downstream target of SHH signaling and its high expression in BCC and MB may be a result of the active SHH signaling in these tumors\textsuperscript{110}. 
Protein degradation and the Ubiquitin-Proteasome System (UPS)

Proteins are constantly produced and degraded in the cell. In order for cells to maintain their biomass, this protein turnover needs to be tightly regulated. The ubiquitin-proteasome system plays the main role in the regulation of protein degradation. This system relies on a three-step process at the end of which the protein which is to be degraded is conjugated with a chain of ubiquitin (Ub) residues\textsuperscript{39}. First, ubiquitin (Ub) residues need to be activated: an ATP molecule is attached to them by E1 enzymes\textsuperscript{111}. Activated Ub can interact with E2 enzymes by forming an E2-Ub complex. Then, after forming a multiprotein complex together with an E3 ligase, which is responsible for specific recognition of the target, the Ub residue is transferred from the E2-Ub complex to the targeted protein\textsuperscript{111}. By repeating the process, a chain of Ubs is attached to the protein, usually leading to its degradation in the 26S proteasome\textsuperscript{112}. However, the Ub chain can be attached to different lysine residues in the protein and can differ in length. Depending on the length of the chain, the conjugated proteins may be recruited in different biological process instead of being degraded\textsuperscript{113}.

\textbf{Figure 4.} Ubiquitin-Proteasome System (UPS). Ubiquitination is an ATP-dependent reaction that involves a concerted effort of an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3). E3 ligases are the source of substrate specificity resulting in ubiquitin conjugation to specific lysine (K) residue(s) on the substrate. The ubiquitination process is counteracted by deubiquitinating (DUBs) enzymes that hydrolyze ubiquitin-protein peptide bonds and recycle ubiquitin molecules.
The E3 ligases are responsible for specifically recognizing the target which has to be ubiquitinylated. Therefore, many different E3 ligases exist\textsuperscript{114}. Most of them have a RING (Really-Interesting New Gene) homology domain which is required for interaction with the E2 enzymes\textsuperscript{39}. E3 ligases can consist either of one subunit (like MDM2\textsuperscript{115}) or of multiple subunits. Well-studied multiple subunit ligases include SCF (SKP1-Cullin1-F-box) and APC/C (anaphase-promoting complex/cyclosome).

**UPS in normal development**

Various developmental pathways need to be strictly controlled in order to function correctly. The UPS system is capable of degrading specific proteins after they have served their function, while others are left intact. The SHH pathway is regulated by SPOP-CUL3\textsuperscript{116} and ITCH\textsuperscript{117} E3 ligases. The WNT pathway requires the degradation of β-catenin by the multiunit E3 ligase SCF\textsuperscript{β-TrCP}. WNT signaling is only active when β-catenin is not phosphorylated rendering the ubiquitin ligase unable to bind to it and target it for degradation\textsuperscript{118}. RTK signaling is also largely regulated by UPS\textsuperscript{119}. The ubiquitin ligase CBL targets multiple tyrosine kinases like EGFR\textsuperscript{120}, HER2\textsuperscript{121}, PDGFRα or PDGFRβ\textsuperscript{122}, FGFR\textsuperscript{123}. The HIF, NOTCH, TGFβ, cell cycle, NFκB pathways make no exception and are, too, regulated by various ubiquitin ligases\textsuperscript{39}.

Important oncogenes and tumor suppressors are regulated by UPS as well. Potent oncogenes like MYC are targets of several ubiquitin ligases: FBXW7, TRUSS, HUWE1, β-TRCP, SKP2 all regulate MYC protein levels\textsuperscript{124}. The tumor suppressor gene p53 is also regulated by the MDM2 ligase\textsuperscript{115}.

**UPS in cancer**

An altered UPS may protect proteins from degradation. This results in higher protein levels than needed and thus problems with the UPS are found in many cancers\textsuperscript{39}. There are mutations in MYC and MYCN which inhibit protein binding to FBW7 ubiquitin ligase (T58A). This mutated MYC (MYCN) can induce medulloblastoma resembling human Group 3\textsuperscript{38,72}. Additionally, wild type MYCN cannot induce glioma or medulloblastoma while the T58A mutant can induce both\textsuperscript{31}. The oncogenic version of EGFR – EGFRvIII – has reduced affinity to its ubiquitin ligase CBL and therefore it is not as rapidly degraded as the wild type EGFR\textsuperscript{125}. An E3 ligase called HAF (Hypoxia Associated Factor) was involved in brain tumorigenesis by targeting the inhibitor of HIF2A and therefore promoting HIF2A stabilization\textsuperscript{126}. 
Present investigations

**Paper I.** Distinct Neural Stem Cell Populations Give Rise to Disparate Brain Tumors in Response to N-MYC

The aim of this study was to investigate if MYCN alone could transform neural stem cells at different stages of their development. We were also interested to see if NSCs from various parts of the brain could induce tumors following MYCN overexpression.

We microdissected cells from three different areas in the mouse brain – SVZ in the forebrain, lower rhombic lip (LRL) of the brain stem and total cerebellum. The mice used were a transgenic Gtv-a line expressing the avian TVA receptor under the control of a GFAP promotor. The animals were sacrificed at two time points in their development – embryonic day 16 (E16) or postnatal day 0 (P0). All of the isolated cells were transfected with RCAS viruses which can only infect cells expressing TVA receptors i.e. GFAP-positive cells. Previous studies had suggested that MYCN could not generate brain tumors alone but that mutationally-stabilized MYCN could immortalize NSCs. As immortalization is a hallmark of cancer we therefore used both wild-type MYCNWT and stabilized MYCNT58A viruses or GFP as a control. The proliferation capacity of all of the infected cells was evaluated in vitro. Both E16 and P0 forebrain and cerebellar cells infected with NMYCT58A viruses showed increased proliferation while brainstem cells isolated from the LRL did not show a difference in proliferation between GFP, NMYCWT or NMYCT58A infected cells. Therefore, we also included E14 cells, which showed an increased proliferation upon NMYCT58A overexpression. SHH pathway dependency upon transfection was also examined. All of the cells from the cerebellum and forebrain were sensitive to cyclopamine (SMO inhibitor) treatment if transfected with GFP, while E16 forebrain and P0 cerebellar cell growth were not inhibited by the drug when infected with NMYCT58A viruses.

Next, we transplanted 100,000 cells from each cell type orthotopically in the mouse brain to study tumor formation abilities. Glioma-like tumors (MG-PNET) formed from forebrain NMYCT58A transformed cells but not from any of the other forebrain cells. Medulloblastoma also developed from the cerebellar cells only after NMYCT58A overexpression. Anaplastic MB/PNET tumors formed from E14 LRL NMYCT58A-expressing cells but no tumor developed from E16 or P0 cells that were infected with either GFP or
NMYC\textsuperscript{WT}. While the gliomas from E16 or P0 cells were similar to each other, the medulloblastoma induced from E16 cells had an SHH molecular signature and that induced from P0 cells resembled Group 4 MB. Further, we followed the expression of the glial marker SOX9 in the transfected cells and in the tumors. We noticed that the SHH dependency in cells correlated with a high SOX9 transcript expression and protein level. Our finding was validated in a patient cohort where SOX9 was highly expressed in SHH and WNT subgroups but not in Group 3 and Group 4. Also, in a series of experiments we showed that if SOX9 is overexpressed in NMYC\textsuperscript{T58A}-expressing P0 cells they induce SHH-dependent tumors instead of Group 4. To summarize, our results demonstrate that the developmental stage of the cells during NMYC transformation may determine the type of tumor that develops. A single transcription factor may alter this, as the overexpression of SOX9 in tumor initiating cells was sufficient to alter the subgroup from SHH to Group 4. Tumors may be induced by mutationally-stabilized NMYC but not from the WT protein.
**Paper II.** FBW7 suppression leads to SOX9 stabilization and increased malignancy in medulloblastoma

In this collaborative effort we aimed to explore the regulation of SOX9 by the UPS. In previous work using proteomic screen, SOX9 was identified as a possible target of FBW7 ubiquitin ligase\(^{129}\). To expand our knowledge on SOX9, we aimed to confirm this interaction and follow its role in medulloblastoma.

We verified the direct binding of FBW7 to SOX9. We further defined a conserved AA sequence in SOX9 (called phosphodegron (CPD)) between Thr236 and Thr240 which was the binding site for FBW7. After phosphorylation by GSK3\(\beta\) this CPD was able to bind to FBW7 leading to SOX9 ubiquitination and degradation in 26S proteasome.

We confirmed that this interaction was relevant in medulloblastoma patients. We studied a TMA (Tissue Microarray) of 142 samples where high RNA was translated to high protein content only when FBW7 levels were low. We also discovered that high FBW7 in patients was correlated to better overall survival and that high levels of the SOX9 protein were correlated to higher M3 stage.

Using mouse models we showed *in vivo* that SOX9 induces migration in MB cells and that this increased migration may be inhibited if FBW7 is induced. Additionally, when FBW7 was induced in the mice, survival was prolonged. These finding were confirmed with *in vitro* experiments. We also wanted to study if stabilized SOX9 would have different impact on its target genes compared to the wild type (WT) protein. Therefore, we used mutation in the CPD (SOX9-T236/240A) and compared it to WT SOX9 using RNAseq. We discovered that the stabilized form is inducing higher expression of EMT genes as well as of genes connected to migration, invasion and metastasis. Therefore we concluded that when SOX9 is stable it is able to induce migratory phenotype in medulloblastoma.

To use our findings for developing a treatment strategy we checked if SOX9 levels change cells’ drug sensitivity. We used cisplatin because it is commonly used in clinics for treatment of MB and we observed that when increasing the levels of SOX9 in cells they become more resistant to treatment. We inhibited the PI3K/AKT/mTOR pathway which was shown to induce SOX9\(^{130}\) and we observed that cells became more sensitive to cisplatin treatment.

Taken together, our work demonstrates how SOX9 is regulated by the UPS. FBW7 was established as the ubiquitin ligase binding and targeting SOX9 for degradation. This regulatory axis contributes to SOX9 stability which is correlated to medulloblastoma malignancy and drug resistance. Finally, we propose that targeting SOX9 stability enhances cells sensitivity to treatment.
**Paper III. Metastasis and tumor recurrence from rare SOX9-positive cells in MYCN-driven medulloblastoma**

The aim of this work was to provide insights on the role of the population of SOX9-positive cells in the SHH-independent medulloblastoma and the molecular signatures that this transcription factor induces in the cells.

In previous studies, by overexpressing SOX9 in NSCs that have been infected with NMYC<sup>T58A</sup>, we observed an inhibition of cell proliferation upon transient high expression of SOX9. However, stable clones with moderate levels of SOX9 promoted tumor incidence instead, and further migrated to the forebrain. This was not the case for cells with low SOX9 levels which always resided in the cerebellum where both cell types were originally injected.

We thus hypothesized that SOX9 may be important for tumor migration, metastasis or recurrence and designed a strategy to study SOX9-positive cells *in vivo*. We used the GTML mouse tetOFF model which recapitulates human NMYC-induced medulloblastoma. GTML animals that developed brain tumors are known to be cured by MYCN depletion from long-term (30 days) dox treatment<sup>33</sup>. Tumor suppression by dox induces a cell cycle block in all tumor cells followed by necrosis or senescence. Interestingly, cells become less apoptotic following dox treatment, which may be due to the fact that MYC proteins are well-known inducers of apoptosis<sup>131</sup>. There are only a few scattered SOX9-positive cells in GTML tumors. Moreover, cells that survive dox administration have higher levels of SOX9 protein. In order to keep SOX9-expressing cells alive during dox treatment, we crossed GTML mice with mice that carry the Sox9rtTA transgene. In this way, when dox food is fed to animals which have developed brain tumors, all the tumor cells die except for the ones with high SOX9 expression. Mice were healthy after several portions of dox food and the luminescence from the tumor disappeared. Upon dox removal however, the tumor returned and relapsed medulloblastoma developed in the forebrain, covering the cerebrum. Cells isolated from such relapses were more migratory (also *in vitro*) when compared to cells from initial GTML. Tumors that recur as distant metastases resemble human Group 3 and Group 4 medulloblastoma. The recurrences in our mouse model were similar to the initial GTML tumors in terms of immunoreactivity and RNA expression of selected markers.
Conclusion and future perspectives

Our knowledge of medulloblastoma has increased during the last few years. Molecular subgroups have divided the tumor into four different entities with different prognosis, different biological characteristics and different drug response. Mouse models have helped discover cells of origin for these tumors. The next challenge in the field would be to change therapies in such a way that these findings become beneficial for the patients. New therapies are also needed, especially for the most aggressive MYC and MYCN amplified tumors. Understanding the disease better would be a valuable asset for defining new targets and treatments.

Research described in this thesis improved the understanding of MYCN-amplified tumors. Our results have proven the importance of MYCN in tumorigenesis. We defined SOX9 as marker of specific cell populations which are more migratory and drug resistant. We further showed how these cells are able to initiate tumor recurrence. Regulation of SOX9 by FBW7 and UPS was established as a way to control SOX9 levels in cells. This regulation appears to lead to increase of SOX9 when FBW7 is downregulated in tumors including medulloblastoma. We also observed negative correlation between MYC proteins and SOX9 in mouse models, patient cell lines and tumor material.

In order to further develop our work, we will try to target signaling pathways overrepresented in SOX9-expressing cells in combination with existing therapies. We need to understand how SOX9-positive cells in the tumors reinitiate brain tumors. Interestingly, SOX9 is induced in cells that reside in the vicinity of blood vessels after dox treatment of GTML tumors in vivo. We would like to determine if these cells connect with endothelial cells in the vascular niche and also study if SOX9-positive cells are more sensitive to MYCN-targeted therapies or radiation therapy. We will also study if SOX9 has a role in inducing cellular senescence as we believe that elevated levels of SOX9 will promote senescence. This could be a way of protecting cells from necrotic or apoptotic cell death.

We have developed inducible lentiviral vectors for overexpressing SOX9 and shRNA directed against SOX9 to suppress it. We hypothesize that these systems could be used for detailed analyses of SOX9 in primary human stem cells as well as in medulloblastoma cells. Different levels of SOX9 can be result in different function, based on our results from mutationally stabilized SOX9. Finally, we aim to further evaluate the potential of the negative corre-
lation of SOX9 and MYC family gene expression. We will study different medulloblastoma subgroups to study if this correlation is true for all the molecular subgroups or is restricted to some. We will also try to use this correlation and our knowledge for SOX9 regulation to try treatments for medulloblastoma based on this regulatory axis. Knowledge and further analysis of active pathways in SOX9 positive cells will also provide us with additional targets in these cells. All *in vitro* treatments which show potential will be evaluated in our mouse model where we will try to block tumor recurrence.
Acknowledgements

This work was carried out at Rudbeck Laboratory, Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University. We acknowledge financial support from the European Research Council, the Swedish Childhood Cancer Foundation, the Swedish Cancer Society, the Swedish Research Council, the Swedish Society of Medicine, the Swedish Brain Foundation, Åke Wiberg’s Foundation, Ragnar Söderberg’s Foundation and the Association for International Cancer Research.

I would like to express my gratitude to the following people who supported me through the years and helped me to prepare this doctoral thesis.

Most importantly, to my supervisor Fredrik Swartling: Thank you for believing in me, for all your patience, guidance and support through the years. Honestly, when I started as your first PhD student, I had some worries how my doctoral studies will go. Now, when my PhD years are close to the end, I know I would never exchange my time in Swartling lab for any other lab in the world.

To my co-supervisor, Bengt Westermark: Thank you for showing me how the scientist should think and reason; for being great inspiration and example and for showing me how one can always ask the right questions.

Thanks to all the people in the neuro-oncology group and especially to the people in FS group: Sara, Anna, Holger, Gabriela, Matko, Sonja, Lotta, Anders, Lisa, Sanna. I cannot imagine how boring would be during the conference evenings if you were not there. Thank you, Olle and Aldwin, for the great collaborations and for the great ideas and enthusiasm. Very much thanks to all our former and current MSc students and people that have been in our lab at least for a while. Special thanks to Kiki and Tiolina for your time in the lab and for doing your MSc projects with me. Hope you learned. I definitely did.

Thank you, Lucy, Doroteya and Frank, for reading and correcting my English (mostly for understanding it) and for all the valuable comments.

Thanks to all the people in my office (even the ones who left) – Gabriela, Antonia, Lucy, E-Jean, Yuan, Smitha, Pratyusha, Yiwen, Tobias and Jelena for the nice conversations, all the laugh, chocolate and other tasty food and your help and support whenever I needed it. Also, although rare, the office lunches were great fun.

Rudbeck lab, IGP and Uppsala in general are fantastic student environment. Thanks to all the people that made my last student years a journey.
Without Anja, Ram, Lucy, Frank, Emma G, Veronica, Eric, Chiara, Miguel, Sara, Sofia, Johanna, Emma Y, Jakob, Marta, Leonor, Isabel, Ines, Maike, Diego, Viktor, Argyris, Matko and many, many others, I wouldn’t have made it through all these years (or they would have been super boring). Thank you for all the quiz nights, game-nights, everything that the PhD student council organized, the fun Alternative Journal Clubs, after works and more than everything, the great scientific discussions at various places all over the world. Special thanks go to Sara, Miguel and Sofia and Emil for all the non-scientific chats and for teaching me more about piñata and other important Swedish and Spanish words and traditions.

To summarize, thanks to all of you working at IGP and Rudbeck lab for making it a great place to do my PhD.

To smoothly change countries, I would like to give my appreciations to all my Bulgarian and semi-Bulgarian friends in Uppsala/Stockholm. It is difficult to follow some very beautiful (and tasty) traditions if there is no one to share the fun with. Luckily, I have a lot of people to share – Doroteya, Pavel, Sandra, Geri, Philip, Dari, Fartash, Simeon, Bojidar, Ioanna, Antonia, Manoj, Vicky, Lofe, Kai, Silvia.

Finally, thanks to all of my friends from back in Bulgaria for always being there when I am back, for staying in touch with me despite the distance and the busy schedules, for critically encouraging all my initiatives, for inspiring me in sometimes ridiculous ways and for asking me all the difficult questions (like what exactly do I do in the lab?). Niki, Mimi, Alex, Iliana, Zori, Veso, Vasilen, Asya, Kalin, Tedi, Rosen, Tito, Rayna, Naso, Pancho among others. Special thanks go to Mimi and Alex for their contribution to this thesis and for making it look much better.

To all my family, but especially to my Mom, Dad and Brother: Thank you for your full support. I would have never been here without you. I wish one day I would be able to create such a nice family as the one in which I was raised.
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Acta Universitatis Upsaliensis

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