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Braun, Sebastian

2012

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*Citation for published version (APA):*

Braun, S. (2012). *Downstream effects of master regulators in two brain diseases*. [Doctoral Thesis (compilation), Stem Cell Center].

*Total number of authors:*

1

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Academic Dissertation

# Downstream effects of master regulators in two brain diseases

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With the approval of the Faculty of Medicine at Lund University,  
this thesis will be defended on September 21, 2011 at 2.30 pm in Segerfalksalen,  
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Organization LUND UNIVERSITY  Department of Laboratory Medicine Lund Strategic Research Center for Stem Cell Biology and Cell Therapy Lund, Sweden	Document name DOCTORAL DISSERTATION	
	Date of issue September 21, 2012	
	Sponsoring organization	
Author(s) Sebastian Braun		
Title and subtitle Downstream effects of master regulators in two brain diseases.		
Abstract <p>In paper one, we investigated how the pharmacological activation and inhibition of the glucocorticoid system affects lifespan and symptoms in a mouse model for RTT. We performed a long-term drug treatment study with the GR activator corticosterone and the GR inhibitor RU486 under which we measured the lifespan and onset of RTT-like symptoms of male <i>Mecp2</i>-null and female <i>Mecp2</i> heterozygous mice in comparison to untreated mutant and to treated and untreated wild-type animals. We could demonstrate that activation of the glucocorticoid hormone system reduces the lifespan of <i>Mecp2</i><sup>-y</sup> mice and the symptom-free lifetime of <i>Mecp2</i><sup>+/-</sup> mice and that treatment with the GR inhibitor RU486 has an opposite effect as it prolongs the lifetime until symptom onset for <i>Mecp2</i><sup>+/-</sup> mice and improves motor functions of <i>Mecp2</i>-null male mice. Our findings provide evidence for the contribution of the glucocorticoid hormone system to RTT motor symptoms and suggests this system as a potential therapeutic target for RTT. In paper two and three, we focused on the molecular events that lead to the development of primary malignant brain tumors. In paper two, we performed a series of transplantation experiments with genetically perturbed cells. We could show that the individual over-expression of potent oncogenes in neural stem/progenitor cells of the same cell pool leads to distinct tumor types. Furthermore, we demonstrated that it is possible to convert one tumor type into another one and that this is determined by the order of genetic events. In a second part of this study we could show a hitherto unknown aspect of AT/RT and rhabdoid tumor biology, an activation of the UPR. We provide experimental evidence that AT/RT and rhabdoid tumor cells with reduced or absent SMARCB1 levels are sensitive toward a further increase in ER stress. In paper three, we studied the PcG protein BMI1 and its effect on neural stem/progenitor cells and tumor formation. We observed a strong promotion of self-renewal, expansion and survival in adult neural stem/progenitor cells upon over-expression of <i>Bmi1</i> in vitro but found it incapable of transforming cells as no tumors developed in intracranial transplantation experiments with <i>Bmi1</i> over-expressing wild-type cells or <i>Trp53</i><sup>-/-</sup> cells. Thus, we assume BMI1 to promote stem cell properties and to act as a facilitator of transforming events induced by other oncogenes. Furthermore, we could identify four novel direct BMI1 target genes whose molecular function may contribute to the known BMI1 effects, thus expanding the BMI1 network. Taken together, the findings presented in this thesis emphasize the key role of master regulators in the pathology of brain diseases and for the development of causal therapies.</p>		
Key words: Rettsyndrom, hjärntumörutveckling, genregulering, neurala stamceller, cellens ursprung, BMI1		
Classification system and/or index terms (if any):		
Supplementary bibliographical information:		Language English
ISSN and key title: 1652-8220		ISBN 978-91-87189-27-2
Recipient's notes	Number of pages	Price
	Security classification	

Distribution by (name and address)

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# Downstream effects of master regulators in two brain diseases



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ISSN 1652-8220

ISBN 978-91-87189-27-2

Lund University, Faculty of Medicine Doctoral Dissertation Series 2012:64

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Printed by Media-Tryck, Lund, Sweden

*To my grandmother, Sieglinde Elise Hagert*



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# ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AT/RT	Atypical teratoid/rhabdoid tumor
BDNF	Brain-derived neurotrophic factor
BMI1	B cell-specific Mo-MLV integration site 1
CDK	Cyclin dependent kinase
ChIP	Chromatin immunoprecipitation
ChIP-seq.	ChIP-sequencing
CML	Chronic myelogenous leukemia
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
CRHR1	CRH1 receptor
DNMT	DNA methyltransferase
Ex	Embryonic day x
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ES	Embryonic stem
EV	Empty vector
FACS	Fluorescence activated cell sorting
FDR	False discovery rate
FGF	Fibroblast growth factor
FKBP5	FK506 binding protein 51
GABA <sub>A</sub> R	Gamma-aminobutyric acid receptor A
GBM	Glioblastoma multiforme

GMP	Granulocyte macrophage progenitor
GR	Glucocorticoid receptor
GSEA	Gene set enrichment analysis
HGFR	Hepatocyte growth factor receptor
HPA	Hypothalamic-pituitary-adrenal axis
HSC	Hematopoietic stem cell
IPC	Intermediate progenitor cell
IRES	Internal ribosomal entry site
LOH	Loss of heterozygosity
LTR	Long terminal repeats
LVW	Lateral ventricle wall
MA	Microarray
MAPK	Microtubule-associated protein kinase
MBD	Methyl CpG-binding domain
MeCP2	Methyl CpG-binding protein 2
MEF	Mouse embryonic fibroblast
Mo-MLV	Moloney murine leukemia virus
MR	Mineralocorticoid receptor
MRT	Malignant rhabdoid tumor
NMDAR	N-methyl-d-aspartate receptor
NSC	Neural stem cell
NSP	Neurosphere
OPC	Oligodendrocyte precursor cell
Px	Postnatal day x
PDGFR	Platelet-derived growth factor receptor
PcG	Polycomb group
PERK	PKR-like ER kinase

PI3K	Phospho-inositol-3-kinase
PNET	Primitive neuroectodermal tumor
POMC	Pro-opiomelanocortin
PP1c	Catalytic subunit of protein phosphatase-1
PRC	Polycomb repressive complex
PRE	Polycomb response element
PVN	Paraventricular nucleus
RG	Radial glial cell
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RTT	Rett syndrome
SGK1	Serum glucocorticoid-inducible kinase 1
SGZ	Subgranular zone
Shh	Sonic hedgehog
SVZ	Subventricular zone
TP53	Tumor protein 53 (human)
TRD	Transcription repression domain
TRP53	Transformation related protein 53 (mouse)
Trx	Trithorax
UPR	Unfolded protein response
VEGFR	Vascular endothelial growth factor receptor

# ORIGINAL PAPERS AND MANUSCRIPTS

- I. Pharmacological interference with the glucocorticoid system influences symptoms and lifespan in a mouse model of Rett syndrome.

Braun S, Kottwitz D, Nuber UA.

*Human Molecular Genetics*, 21:1673-1680, 2012.

- II. Definition of genetic events directing the development of distinct types of brain tumors from postnatal neural stem/progenitor cells.

Hertwig F, Meyer K, Braun S, EK S, Spang R, Pfenninger CV, Artner I, Chen X, Biegel JA, Judkins AR, Englund E, Nuber UA.

*Cancer Research*, 72(13):3381-3392, 2012.

- III. Identification of novel BMI1 target genes in neural stem/progenitor cells.

Hertwig F\*, Braun S\*, Nuber UA.

\* equal contribution

*Manuscript*

# SUMMARY

The human brain is an exceptional organ- both in terms of abilities and complexity. Its remarkable achievements, like cognition, creativity and emotions, are realized by a unique cellular network in which more than one hundred billion neurons, supported by surrounding glial cells, interact and adapt to input from the environment. Understanding the architecture and functioning of the brain and its enormous diversity of cells remains a scientific challenge. The complexity of the human brain is accompanied by a high vulnerability that makes it susceptible to regulative alterations and complicates the treatment of brain diseases.

Glial cells and neurons differentiate from neural stem and progenitor cells within a frame of developmental programs that coordinate a correct temporal and spatial gene expression. The demand on this transcriptional control system is high: it must assure the proper development and plasticity of the embryonic and neonatal brain as well as the maintenance of a mature neuronal network of the older brain. While neurogenesis persists lifelong in certain brain regions, different levels of proliferating cells, immature and committed cells, are required. Therefore, gene expression regulation must be adaptive, efficient and exact.

A key feature of the cellular transcription regulation is its strict hierarchical organisation in which so called master regulators control numerous downstream target genes. The consequences for the CNS are therefore particularly dramatic if the transcription of such master regulator genes standing on top of this regulatory hierarchy is altered: any change of their physiological expression or activation pattern will disturb the correct spatial and temporal gene expression and ultimately cause an altered cellular development, proliferation and functioning of the embryonic, juvenile or adult brain.

This thesis is dealing with two severe types of brain diseases, Rett Syndrome (RTT) and malignant brain tumors, which are related to an altered structure or expression of master regulators in the brain. The main focus is on the resulting altered expression of respective downstream target genes.

RTT is a severe neurological disorder and the majority of cases are caused by mutations in a single gene, *MECP2*. Its protein product, methyl-CpG binding protein 2 (MeCP2), mediates chromatin modifications and acts as a modulator of gene expression. In paper one, which is based on the finding that glucocorticoid-regulated genes are direct MeCP2 targets, we investigated the functional implication of the glucocorticoid hormone system in RTT.

Papers two and three are dealing with the development of primary malignant brain tumors from postnatal neural stem/progenitor cells. In paper two we investigated how certain genetic perturbations of murine neural stem/progenitor cells direct the development of distinct brain tumor types and we present a hitherto unknown involvement of the unfolded protein response (UPR) in atypical teratoid/rhabdoid (AT/RT) and malignant rhabdoid tumor (MRT) biology that is associated with an inactivation of the *SMARCB1* gene.

BMI1 is a Polycomb group (PcG) protein and involved in both neural and brain tumor development. In paper three we show that over-expression of *Bmi1* increases self-renewal and proliferation in neural stem/progenitor cells and leads to decreased cell death. Moreover, we present four novel direct BMI1 target genes whose downregulation is likely to contribute to the described BMI1 effects.

The aim of this thesis is to contribute to a better understanding on how downstream consequences of altered master regulators lead to pathological cellular changes underlying the development of certain brain diseases.

# INTRODUCTION

## Rett syndrome

RTT is a neurological disorder that affects mainly girls with an incidence of one in 10.000 (Hagberg et al., 1983). Typically, the disease manifests at an age of 6-18 months and the patients may even achieve the ability to walk and to speak a few words. Early symptoms of the beginning disease are deceleration of head growth that turns into microcephaly followed by a generally retarded development, weight loss, muscle hypotonia, disturbed motor coordination and ataxia. Later on, patients lose purposeful hand use and develop stereotypic hand wringing gestures, breathing anomalies and seizures that may range from easily controlled to epilepsy. Moreover, autistic features like social withdrawal and complete loss of language are common. Patients may reach an age of 70 years or more but suffer from osteopenia, scoliosis and rigidity and may develop cardiac abnormalities and even parkinsonian features in a late motor deterioration phase (clinical features reviewed in (Chahrour et al., 2007)).

RTT was first described by the Viennese pediatrician Andreas Rett, who observed a similar, unusual behavioral pattern among his female patients, in 1966 (Rett, 1966), but received full scientific attention only decades later when described by the Swedish neurologist Bengt Hagberg in the 1980s (Hagberg et al., 1983). Although RTT was then recognized as a distinct entity and assumed to be genetically determined, the molecular background of the disease remained unclear until 1999 when the syndrome could be linked to mutations in methyl-CpG-binding protein 2 (MeCP2) (Amir et al., 1999).

### **Structure and function of MeCP2**

The mechanism of DNA methylation-induced gene repression is involved in fundamental biological processes like genomic imprinting and the control of tissue-specific gene expression. It also plays a role in cancer development through silencing of tumor suppressor genes. Though DNA methylation itself might represent a route to transcriptional repression, preventing the binding of the transcriptional machinery, the major part of the DNA methylation effect seems to be due to chromatin condensation subsequent to methylation, which is mediated by methyl CpG-binding proteins (Bird et al., 1999).



One member of this family is MeCP2, a nuclear protein encoded by an X-chromosome linked gene. MeCP2 consists of a methyl CpG-binding domain (MBD), a transcriptional repression domain (TRD), a C-terminal domain and two nuclear localization signals. Due to alternative splicing of exon 2, two isoforms of the human protein can result, which differ only slightly in their N-termini. MeCP2 is generally categorized as an "intrinsically disordered" protein that does not fold into classical secondary structures like alpha helix or beta sheet (Hansen et al., 2010).

MeCP2 was originally identified as a transcriptional repressor that selectively interprets DNA methylation marks (Lewis et al., 1992), an ability conferred by the MBD of the protein. Moreover, MeCP2 possesses multiple non-specific binding sites (Hansen et al., 2010) and also binds to unmethylated DNA with an only three-fold lower affinity than for methylated DNA (Fraga et al., 2003). This property presumably allows the protein to bind weakly to any site, to migrate along the DNA and to track methylated sequences *in vivo* more efficient (Georgel et al., 2003; Halford et al., 2004).

The MeCP2 TRD was found to be involved in the recruitment of co-repressors and chromatin remodelling complexes such as the co-repressors NcoR, Sin3A, histone deacetylases I and II and the catalytic component of the chromatin remodelling complex SWI/SNF, Brahma (Guy et al., 2011; Harikrishnan et al., 2005; Jones et al., 1998; Nan et al., 1998). The discovery of this cooperation suggested a link between DNA methylation, chromatin modification and gene silencing that was further supported by *in vitro* studies showing that MeCP2 at the molecular level condenses unmethylated or methylated chromatin fibers into highly compact structures (Georgel et al., 2003; Nikitina et al., 2007). Based on these facts, MeCP2 was for a long time assumed to be exclusively acting as a transcriptional repressor. This concept had to be reconsidered after a gene expression study revealed that MeCP2 can function as an activator of transcription, too, up- or down-regulating the expression of a wide range of genes (Chahrour et al., 2008).

The multifunctionality of MeCP2 is emphasized by the fact that in addition to binding chromatin, DNA and other proteins, it also interacts with the RNA-binding protein Y box-binding protein 1 (YB1) and might modulate RNA splicing *in vivo*. MeCP2 was shown to regulate splicing *in vitro* and aberrant alternative splicing patterns were found in a mouse model of RTT (Young et al., 2005). Moreover, MeCP2 was found to interact *in vivo* with mRNAs from genes known to be expressed when their promoters are associated with MeCP2 (Long et al., 2011).

## **Expression of MeCP2**

Studies that analyzed the distribution of MeCP2 in human and murine brain tissue revealed that it is widely expressed and that the highest protein levels occur in brain,

particularly in mature, postmigratory neurons (Akbarian et al., 2001; Balmer et al., 2003; Jung et al., 2003; Shahbazian et al., 2002). In mouse brain, first transcripts of *Mecp2* are detectable in the spinal cord and brainstem around day E12, followed by expression in other brain regions. Generally, protein levels are low during embryogenesis and increase progressively during the postnatal period of neuronal maturation. The cortical expression pattern follows an inner-to-outer sequence (Chahrour et al., 2007; Shahbazian et al., 2002).

These observations, together with the finding that expression of MeCP2 in postmitotic neurons is sufficient to rescue the *Mecp2*-null mouse phenotype (see below) (Luikenhuis et al., 2004), led to the assumption that the pathology of RTT is exclusively related to neurons. However, Ballas and colleagues found low levels of MeCP2 in astrocytes and reported a deleterious effect of *Mecp2*-null astrocytes on *Mecp2*-null as well as wild-type neurons *in vitro* (Ballas et al., 2009) that has been confirmed by other studies (Maezawa et al., 2010; Maezawa et al., 2009). Moreover, a recent publication presented evidence for the significance of MeCP2 in microglia as wild-type *Mecp2*-expressing microglia within the background of *Mecp2*-null male mice alleviated certain features of the disease pathology, improving breathing patterns and locomotor activity and increasing body weight as well as lifespan of the mice (Derecki et al., 2012).

## **Pathology of RTT**

The major cases of RTT are caused by different genetic aberrations in *MECP2* that include missense and nonsense mutations, insertions, deletions and splice site variations that occur throughout the gene (Matijevic et al., 2009; Na et al.). It has been shown that there are 8 common *MECP2* mutations that account for about 70% of all RTT cases and lead to MeCP2 loss of function due to truncated, unstable or abnormally folded proteins (Bienvenu et al., 2006). Most mutations arise *de novo* in the paternal germ line, involving C to T transitions at CpG dinucleotides (Trappe et al., 2001). High levels of MeCP2 may result in phenotypes similar to classical RTT and have been observed in patients carrying duplications of the entire *MECP2* locus (Archer et al., 2006), thus underlining the importance of a fine-tuned *MECP2* expression.

In females, the choice of which X chromosome is active is usually random with half of the cells having the maternal and the other half of the cells having the paternal X chromosome active while the second X chromosome is inactivated. Since *MECP2* is located on the X chromosome, a female patient with a *MECP2* mutation is typically mosaic whereby half of the cells express the wild-type and the other half express the mutant *MECP2* allele (Chahrour et al., 2007). This is one explanation for the milder phenotype in female as compared to male patients. Male patients with mutations in

*MECP2* can be divided in three groups: affected males with classical mutations die in early infancy because of a central breathing failure. Male patients with somatic mosaicisms for *MECP2* mutations or Klinefelter syndrome develop symptoms similar to RTT in females while the third group of males is heterogeneous in phenotype and carries mutations that are inherited from their mothers and have never been found in females with RTT (Bienvenu et al., 2006).

Deficiency for MeCP2 causes a reduced total size of the human brain, with the cerebral hemispheres being more affected than the cerebellum. Alterations in brain volume have been found in the prefrontal, posterior frontal and anterior temporal regions of the cerebral cortex (Reiss et al., 1993; Subramaniam et al., 1997). At the cellular level, neuronal soma are smaller and cells appear more densely packed (Armstrong, 2005). Neurons of the RTT affected human brain exhibit reduced dendritic branching and reduced dendritic spine density (Belichenko et al., 1994; Chapleau et al., 2009). It is noteworthy that no degeneration, atrophy or inflammation has been found in the MeCP2 deficient brain, indicating that a neurodegenerative process is not involved in RTT (Jellinger et al., 1988; Reiss et al., 1993).

In studies derived from mouse models for RTT, defects in spine morphology, an abnormal number of axons and a defect in axonal targeting have been detected (Belichenko et al., 2009; Chao et al., 2007). Analysis of the murine *Mecp2*-null brains revealed evidence for defects of neurotransmission in RTT as altered levels of neurotransmitters such as glutamate and biogenic amines as well as changes in the abundance of neurotransmitter receptors like N-methyl-d-aspartate receptor (NMDAR) and gamma-aminobutyric acid receptor A (GABA<sub>A</sub>R) were detected in murine *Mecp2* knockout brains (Armstrong, 2005; Asaka et al., 2006; Guy et al., 2011; Medrihan et al., 2008). Reduced levels of serotonin, adrenaline and dopamine were found in brain tissue of *Mecp2*-null mice (Ide et al., 2005; Isoda et al., 2010; Samaco et al., 2009; Santos et al., 2010) and associated with expression defects of tyrosine hydroxylase and tryptophan hydroxylase 2 in brain stem, substantia nigra and raphe nuclei tissue (Samaco et al., 2009; Taneja et al., 2009; Viemari et al., 2005). Synaptic dysfunction was detected by a shift in the excitatory/inhibitory balance of postsynaptic currents found in hippocampus and cortex of RTT mouse models (Chao et al., 2007; Dani et al., 2005; Medrihan et al., 2008; Zhang et al., 2008) and an altered long-term potentiation in the hippocampus of symptomatic MeCP2 deficient mice (Asaka et al., 2006; Guy et al., 2007; Weng et al., 2011). Together these studies indicate that loss of MeCP2 disturbs synaptic function in certain regions of the murine brain, thereby disrupting the efficiency of neuronal networks.

## The role of MeCP2 in brain development and maintenance

Much insight on the nature, progress and pathology of RTT as well as the function of MeCP2 could be gained by the generation of mice that are null or heterozygous for *Mecp2*. *Mecp2*-null males showed a clear RTT-like phenotype, displaying neurological symptoms such as irregular breathing, hindlimb claspings, gait and tremor (Guy et al., 2001) as well as reduced brain size and smaller, more densely packed neurons in hippocampus, cortex and cerebellum (Chen et al., 2001). Embryonic deletion of exon three alone or together with exon four from the *Mecp2* locus applying a Cre-recombinase expressed from the Nestin-promoter proved that MeCP2 dysfunction in the brain is sufficient to cause the disease (Chen et al., 2001; Guy et al., 2001). Moreover, MeCP2 seemed to be dispensable for early growth and differentiation as the transgenic mice develop normal brain structures until birth (Chen et al., 2001; Guy et al., 2011; Guy et al., 2001), *Mecp2*-null neuronal precursors are able to differentiate into various neuronal and glial cell lineages (Kishi et al., 2004) and the highest levels of MeCP2 are found exclusively in postmitotic neurons (Jung et al., 2003).

The onset of clear RTT symptoms in humans at the age of 6-18 months coincides with a period of widespread synaptogenesis in the human brain (Huttenlocher et al., 1997), thus pointing toward a disturbed formation of synapses and neural networks in the MeCP2 deficient brain that has been documented for RTT (Fukuda et al., 2005; Johnston et al., 2001). The relatively late symptom onset in female mice heterozygous for *Mecp2* nevertheless indicates that MeCP2 is necessary to maintain a fully functional neuronal network even after brain development has finished (Guy et al., 2011). This assumption is supported by a recent study from McGraw and colleagues that demonstrates a recapitulation of the germline knockout phenotype if *Mecp2* is deleted in adult mice (McGraw et al., 2011).

A central question in RTT research is if the absence of functional MeCP2 causes irreversible abnormalities in the developing brain or if the physiological brain function can be restored even after the onset of symptoms at postnatal stages. Guy and colleagues could show that a gradual restoration of MeCP2 in a three to four weeks old RTT mouse model increases lifespan, reverses deficits in motor coordination and respiratory function and improves neurological functions (Guy et al., 2007). These important findings are supported by two other studies demonstrating that the RTT phenotype can be partially rescued if MeCP2 is restored in postnatal neurons (Giacometti et al., 2007; Luikenhuis et al., 2004).

## MeCP2 target genes and therapeutical approaches

The finding that a restoration of MeCP2 in knockout mice can reverse major symptoms of the RTT phenotype raised hope for the development of a treatment of this disease. Gene therapy might represent one approach. However, it comes with several restrictions, a major one being the fact that the level of *Mecp2* expression is crucial for the rescue of the RTT phenotype. With the help of mouse models it has been shown that over-expression of *Mecp2* in postmitotic neurons (Luikenhuis et al., 2004) as well as abrupt restoration of MeCP2 in brain (Giacometti et al., 2007) results in the onset of motor dysfunction (swaying, tremor, gait ataxia) and cannot prevent death of the mice, respectively. Given that *Mecp2* expression levels might differ significantly between cell types, brain regions and even developmental phases and that female patients exhibit a mosaic expression pattern, correct delivery and dosage of MeCP2 is a big challenge (reviewed in (Gadalla et al., 2011)).

Therefore, a pharmaceutical treatment that focuses on genes whose altered expression is a consequence of dysfunctional MeCP2 and which are deregulated in RTT patients, might represent an alternative therapeutical approach. The advantages of a pharmaceutical strategy over a gene therapy are obvious: it would be less invasive, easier to control and better to dose.

The precondition for a target gene-focused treatment of RTT is a detailed knowledge about the regulatory function of MeCP2. Early expression studies that aimed at identifying upregulated genes in brain tissue of *Mecp2*-null mice failed to reveal obvious expression changes, and were interpreted as MeCP2 either influences transcription only modestly, thus “fine-tuning” gene expression, and/or acts differently in individual tissue and cell types (Chahrour et al., 2007; Tudor et al., 2002). However, subsequent studies could identify several direct target genes that might contribute to the RTT phenotype (listed in Table 1).

Brain-derived neurotrophic factor (*Bdnf*) was among the first genes that were associated with RTT. BDNF is as growth factor involved in neurogenesis, neuronal maturation and survival and plays a role in synaptogenesis, learning and memory. BDNF is dysregulated in a number of neurological disorders including epilepsy (Binder et al., 2004). Chen and colleagues found that MeCP2 represses the transcription of *Bdnf* *in vitro* in an activity-dependent manner, by dissociation from the *Bdnf* promoter upon membrane depolarization (Chen et al., 2003). However, Chang et al. could demonstrate that BDNF levels are decreased rather than increased in *Mecp2*-null mice and that *Bdnf* deletion in postmitotic neurons of mice led to the onset of RTT-like symptoms (Chang et al., 2006). After speculations that MeCP2 deficiency leads to an overall reduced neuronal activity, which might then indirectly cause decreased *Bdnf* expression, a possible explanation was provided by Charour and

colleagues who showed that MeCP2 through interaction with CREB1 can act as an activator and found *Bdnf* up- or downregulated in hypothalamic tissue of *Mecp2* over-expressing or *Mecp2*-null mice, respectively (Chahrouh et al., 2008). Based on these findings, CX546, a positive modulator of AMPA receptors, which are known to enhance BDNF levels, was tested in *Mecp2*-null mice and could improve respiratory function to normal breathing patterns (Ogier et al., 2007). A follow-up study showed strong reduction of BDNF in the nucleus tractus solitarius, a brain stem nucleus that is important for cardiorespiratory control, of *Mecp2*-null mice, resulting in a hampered synaptic signalling that could be reversed by application of exogenous BDNF (Gadalla et al., 2011; Kline et al., 2010).

IGF1 is another growth factor involved in neuronal maturation and synaptic plasticity. Elevated expression levels of the direct MeCP2 target gene *Igf1p3* have been found in both human RTT patients and *Mecp2*-null mice (Itoh et al., 2007). This change may lead to a decreased IGF1 signalling. Treatment of *Mecp2* knockout mice with the tripeptide of IGF1 was highly beneficial for the mice as it restored brain weight to wild-type levels and led to an increased lifespan, improved locomotor activity and improved cardiac as well as respiratory functions (Tropea et al., 2009). However, the benefit was only transient as all mice developed RTT-like symptoms and showed reduced survival compared to the control group. Recombinant IGF1 is currently being tested in initial phase clinical trials in RTT patients (Gadalla et al., 2011).

Other pharmacological interventions being tested are based on the deteriorated signal transmission in *Mecp2*-null mice (see above). Since reduced bioamine levels in the brain of both RTT patients and mouse models have been found, desipramine, an antidepressant that boosts noradrenaline signalling, has been tested in *Mecp2*-null mice and delayed the onset of breathing abnormalities as well as doubled the lifespan when given to symptomatic mice (Roux et al., 2007; Viemari et al., 2005). Based on the reported dysfunction of the cholinergic system in the RTT brain (Wenk et al., 1996), *Mecp2*-null mice were also fed with a diet rich in choline, yielding only subtle improvements in locomotor and motor tasks without improvements in disease progression or survival (Nag et al., 2007). Studies that focused on the major neural excitatory transmitter, glutamate, and the major neural inhibitory transmitter, GABA, achieved alleviation of certain RTT-like symptoms when treating *Mecp2*-mutant mice, such as better synaptic plasticity or improved respiration and motor functions (Abdala et al., 2010; Voituron et al., 2009; Voituron et al., 2010). Initial phase clinical trials with certain neurotransmitters have been started.

The pharmacological studies using RTT mouse models that have been conducted so far showed that there may be a possibility for a causal and effective RTT treatment. It has, however, become clear that the development of a future pharmacological therapy will be challenging and likely comprise a set of drugs to treat the main aspects of this complex disease.

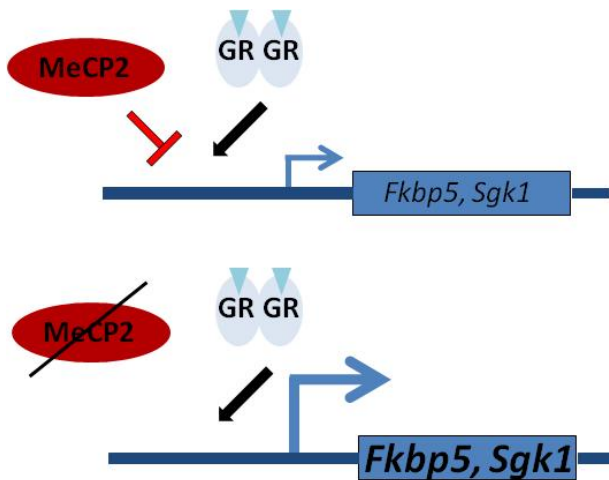
**Table 1. Direct MeCP2 target genes that are dysregulated in the brains of RTT mouse models with impaired MeCP2 function.**

Gene	Function	Applied mouse model/cell type/tissue	Reference
<i>Bdnf</i>	neural development	cultured E18 cortical rat neurons	(Chen et al., 2003)
<i>Fkbp5</i>	hormone signalling	<i>Mecp2<sup>-/-</sup></i>	(Nuber et al., 2005)
<i>Sgk1</i>	hormone signalling	<i>Mecp2<sup>-/-</sup></i>	(Nuber et al., 2005)
<i>Uqcrc1</i>	mitochondrial respiratory chain	<i>Mecp2<sup>-/-</sup></i>	(Kriaucionis et al., 2006)
<i>Crh</i>	neuropeptide	<i>Mecp2<sup>308/-</sup></i>	(McGill et al., 2006)
<i>Id1-3</i>	neuronal transcription factors	cultured human SH-SY5Y neuronal cells transfected with a methylated oligonucleotide decoy to block MeCP2 binding, <i>Mecp2<sup>-/-</sup></i>	(Peddada et al., 2006)
<i>Igfbp3</i>	hormone signalling	RTT patient brain material, <i>Mecp2<sup>-/-</sup></i>	(Itoh et al., 2007)
<i>Fxyd1</i>	ion channel regulator	RTT patient brain material, <i>Mecp2<sup>-/-</sup></i>	(Deng et al., 2007)
<i>Gad1/2</i>	signal transmission	<i>Mecp2<sup>-/-</sup></i>	(Chao et al., 2010)

### **RTT and its relation to the glucocorticoid hormone system**

In order to identify genes that are dysregulated upon MeCP2 loss of function, Nuber and colleagues performed a microarray analysis using total RNA from symptomatic *Mecp2<sup>-/-</sup>* and wild-type littermate control brains (Nuber et al., 2005). 11 differentially expressed genes were found, five of which are known to be regulated by glucocorticoids. Two of them, *Fkbp5* and *Sgk1*, were studied in more detail. Importantly, subsequent analysis of total brain RNA from pre-symptomatic, early symptomatic and late symptomatic mice revealed that *Fkbp5* and *Sgk1* are misregulated already before symptoms occur, thereby ruling out that the deregulation

occurred as a secondary consequence of symptom-regulated changes. Furthermore, no elevated basal plasma glucocorticoid levels, possibly leading to an *Fkbp5* and *Sgk1* over-expression, were detected in *Mecp2<sup>fl/y</sup>* mice. It could be demonstrated that MeCP2 directly binds to glucocorticoid receptor (GR) independent sites of the *Fkbp5* and *Sgk1* promoters (Figure 1). In conclusion, the study expanded the view on RTT, presenting evidence for a function of MeCP2 as a modulator of glucocorticoid-inducible gene expression. Taking into consideration the deleterious effects of an exaggerated glucocorticoid-exposure, it raised the possibility that disruption of MeCP2-dependent regulation of stress-responsive genes contributes to the symptoms of RTT.



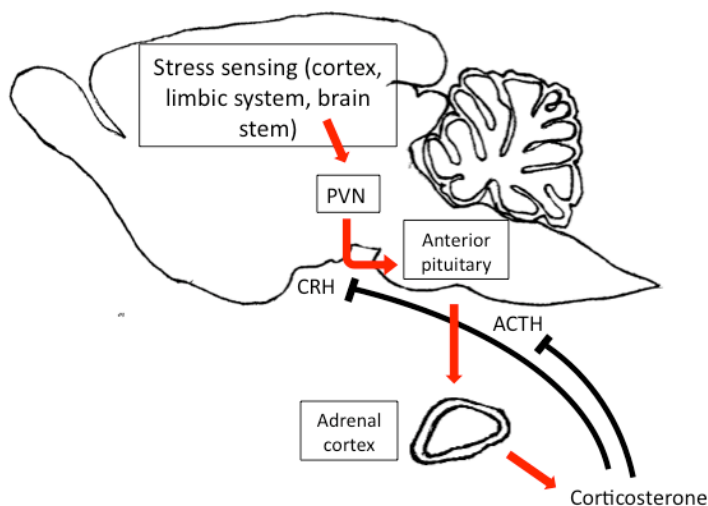
**Figure 1. Model of the MeCP2-mediated repression of the glucocorticoid-regulated genes *Fkbp5* and *Sgk1*.**

### **The glucocorticoid hormone system**

The term stress has classically been defined as the threat of homeostasis by physical and psychological events, so-called stressors, that provoke a non-specific body reaction, the stress response, aiming at reinstating homeostasis (Selye, 1950). The stress concept has been the object of critical discussion under the last decades and more recently, it has been suggested to redefine the term stress to conditions where an environmental demand exceeds the natural regulatory capacity of an organism (He et al., 2008).



The mammalian response to stress involves an interaction of the hypothalamus, pituitary and adrenal cortex, the hypothalamic-pituitary-adrenal axis (HPA) (de Kloet et al., 2005). Upon stress perception, parvocellular neurons of the hypothalamus secrete the neuropeptide corticotropin-releasing hormone (CRH) into the portal vessel system (Figure 2). CRH binds to the CRH1 receptor (CRHR1) in the anterior pituitary gland and induces the synthesis of pro-opiomelanocortin (POMC). POMC is a precursor polypeptide that undergoes post-translational processing via cleavage through tissue-specific prohormone convertases and is converted into different bioactive peptides. In the pituitary gland, POMC is processed to melanotropin, involved in the production regulation of melanin, by melanotropic cells of the intermediate lobe while in the anterior part it is processed to adrenocorticotrophic hormone (ACTH). ACTH finally stimulates the adrenal cortex to secrete glucocorticoids (cortisol in humans, corticosterone in rodents) into the blood. Glucocorticoids can function via a rapid, non-genomic pathway, directly affecting cellular excitability in subfields of the hippocampus, and via a genomic pathway that is slower and longer lasting (Joels, 2008). The genomic pathway of glucocorticoid hormones is mediated by a receptor system consisting of the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) that are co-expressed in the limbic system of the brain. While MRs bind glucocorticoids with high affinity and are already occupied at low glucocorticoid levels, GRs, that are expressed ubiquitously in neurons and glia, have only one tenth of the affinity of MRs and become fully activated when glucocorticoid levels rise significantly (de Kloet et al., 1999).



**Figure 2. Basic HPA.**

Stress perception triggers a cascade in which first the paraventricular nucleus (PVN) of the hypothalamus is stimulated and produces CRH. This hormone is transported to the pituitary where it triggers the secretion of ACTH into the blood stream. When ACTH finally reaches the adrenal gland it leads to the release of corticosterone (rodents) or cortisol (human). Heightened levels of glucocorticoids inhibit further production of CRH and ACTH, turning off the HPA response via feedback inhibition (figure adapted from de Kloet, 2005).

GRs/MRs are assembled in a multiprotein HSP90/HSP70-based chaperone complex that regulates steroid binding: GR-HSP90 interaction allows the opening of a ligand-binding cleft that is accessed by glucocorticoids (Odermatt et al., 2009; Pratt et al., 2006; Pratt et al., 1997). Upon ligand binding, GRs/MRs are phosphorylated (Ismaili et al., 2004), dimerize and a transformation of the GR/MR takes place that is required for dynein-dependent translocation from the cytosol to the nucleus (Galigniana et al., 2004; Pratt et al., 2006). GR/MR-homodimers interact with genomic DNA and bind to glucocorticoid response elements of certain promoter regions. It has also been shown that the two receptors can form heterodimers and that GR-monomers interact with transcription factors like NF- $\kappa$ B or other proteins (De Bosscher et al., 2003; Odermatt et al., 2009). The HSP90 chaperone machinery plays a critical role in GR/MR movement to such transcription regulatory sites (Pratt et al., 2006). MR and GR regulate the transcription of genes that are involved in the

control of receptors, ion channels, ionotropic receptors and ion pumps by attracting co-activators or co-repressors (de Kloet et al., 2005). Despite the fact that both MR and GR can be activated by glucocorticoids and bind to the same DNA sequences, the two receptors can exert distinct transcriptional responses due to the differential recruitment of co-activating/repressing proteins (Odermatt et al., 2009).

The termination of the stress reaction is ensured by a feedback inhibition. If the stressor is no longer present, a recovery phase is started and circulating glucocorticoids inhibit the synthesis of further CRH or ACTH in the hypothalamus and the pituitary, respectively. This reactive feedback results due to a GR-mediated blockade of a stress-induced HPA activation. GRs mediate the activation of excitatory input to the paraventricular nucleus. Moreover, an inhibitory effect of hippocampal MRs on the HPA that is modulated via GRs has been described and emphasizes the importance of a balance between MR- and GR-mediated effects involved in HPA regulation (De Kloet et al., 1998).

A situation in which mostly MRs but only few GRs are activated is associated with small  $Ca^{2+}$  currents, reduced spike-frequency accommodation, stable responses to repeated stimulation of glutamatergic pathways and small responses to biogenic amines, thus maintaining homeostasis (de Kloet et al., 1999). Activation of GRs in addition to MRs after stress results in enhanced  $Ca^{2+}$  influx, stronger spike-frequency accommodations and marked responses to biogenic amines. Thereby, cellular activity after stressful situations is reduced and the recovery of a disturbed homeostasis is facilitated. The coordinated MR- and GR-mediated effects serve to select the most appropriate response to the actual situation of the organism. Under conditions that lead to chronic glucocorticoid exposure, this balance between MR and GR mediated effects can be turned into maladaptation that might lead to a state of vulnerability including atrophy of hippocampal cells, reduced neurogenesis, altered monoaminergic signalling, reduced synaptic plasticity and impaired learning ability (de Kloet et al., 2005; de Kloet et al., 1999).

### **Effects of a chronic exposure to glucocorticoids**

Persisting exposure to high concentrations of glucocorticoids can become harmful and might lead to depression, abdominal obesity, osteoporosis and cardiovascular problems. Hypercortisolaemia might occur as a consequence of pituitary tumors (Cushing disease) or after long-term treatment with glucocorticoids (Cushing syndrome). Certain stress-induced processes, like allocation of ATP by activation of the gluconeogenesis, are useful if time restricted but may deteriorate homeostasis when becoming chronic.

The negative effects of chronically elevated glucocorticoid levels on the mammalian brain have been well documented in the literature. Long-term exposure to

glucocorticoids is detrimental to the developing brain in general (reviewed in (De Kloet et al., 1988)) as it inhibits dendritic growth and synaptic maturation (Kumamaru et al., 2008). Chronic stress or glucocorticoid exposure also negatively affect the adult brain, particularly the GR rich hippocampus (Sapolsky, 1985), leading to general neuronal atrophy. Numerous studies report alterations in dendritic morphology of neurons like a decreased number of apical dendritic branch points and apical dendritic length for different parts of the hippocampus (Watanabe et al., 1992; Wellman, 2001; Woolley et al., 1990). These changes are functionally associated to deficits in learning (Garcia, 2001; Lathé, 2001) and impaired memory (He et al., 2008) as well as reduced synaptic plasticity in general (de Kloet et al., 1999). Furthermore, it has been shown that chronic glucocorticoid administration reorganizes dendritic morphology in the cortex, which might further contribute to stress-induced changes in cognition (Wellman, 2001).

It is remarkable that brain-specific effects of chronic stress exposure like neuronal atrophy and reduced synaptic plasticity are well-described symptoms of RTT, too (Armstrong, 2005; Belichenko et al., 1994; Belichenko et al., 2009; Na et al., 2012). Osteopenia, a non-neurological aspect of hypercorticotropism (Compston, 2010), has also been described in RTT (Hofstaetter et al., 2010; O'Connor et al., 2009).

RTT patients often show increased alertness and heightened anxiety (Mount et al., 2003; Sansom et al., 1993). The behaviour of an RTT mouse with an *Mecp2* truncating mutation in open field testing indicated higher levels of overall anxiety, too (Shahbazian et al., 2002). In order to find out more about anxiety as a component of the behavioral RTT phenotype, McGill and colleagues performed physiological tests with the mouse model generated by Shahbazian et al. and revealed an abnormal stress response of these mice (McGill et al., 2006). Further investigation of the HPA axis showed elevated serum glucocorticoid levels upon stress as well as an over-expression of *Crb*, which is directly regulated by MeCP2, in the hypothalamus, the central amygdala and the stria terminalis (McGill et al., 2006).

In conclusion, the similarities between the chronic stress phenotype and RTT and the finding that the HPA axis is altered in a mouse model of RTT point toward a relation between the stress system and this disorder; the fact that MeCP2 directly targets the glucocorticoid-regulated genes *Fkbp5* and *Sgk1* (Nuber et al., 2005) indicates that downstream effects of glucocorticoids/GRs are activated in the absence of functional MeCP2.

### ***Fkbp5* and *Sgk1* - two stress-related target genes of MeCP2**

#### *FKBP5*

Several studies suggest the HSP90 co-chaperone FK506 binding protein 51 (FKBP5) as an important functional regulator of the GR-complex (Grad et al., 2007; Pratt et

al., 1997). FKBP5 binds to HSP90 during maturation of the GR-complex, conferring a lower binding affinity for glucocorticoids (Wochnik et al., 2005). After hormone binding and activation of the GR, FKBP5 is substituted by its counterpart, FKBP4, which recruits dynein into the complex, thereby allowing the nuclear translocation of the complex and subsequent transcriptional regulation (Davies et al., 2002; Wochnik et al., 2005). Moreover, expression induction of *Fkbp5* by steroids represents an intracellular, ultra-short negative feedback loop that reduces GR sensitivity (Vermeer et al., 2003). Due to these functions, FKBP5 is an important mediator of the stress response and relevant for mood and anxiety disorders: an overshooting induction of *Fkbp5* following steroid hormone release in response to stress may impair the negative feedback of the system, thereby prolonging elevated glucocorticoid levels (Binder, 2009). Such maladaptive stress responses render individuals vulnerable and prone to psychiatric diseases. Over-expression of *FKBP5* has been associated to unipolar and bipolar depression as well as posttraumatic stress disorders (reviewed in (Binder, 2009)).

### *SGK1*

The serum glucocorticoid-inducible kinase-1 (SGK1) is ubiquitously expressed in mammals with varying transcription levels in different cell types. Along with its related isoforms SGK2 and SGK3 it regulates ion channel activity, transport and transcription (reviewed in (Lang et al., 2006; Lang et al., 2010)). *Sgk1* transcription is controlled by a variety of hormones, including glucocorticoids, as well as insulin, growth factors,  $\text{Ca}^{2+}$  and NO. It mediates many different cellular functions like cell volume, certain enzymes and transcription factors, cellular transport, hormone release, neuroexcitability, inflammation, cell proliferation, apoptosis and electrolyte homeostasis (Lang et al., 2006). Moreover, SGK1 is considered to play a role in long-term memory formation as it has been shown to facilitate memory consolidation (Tsai et al., 2002) and *Sgk1* inactivation impaired the expression of long-term potentiation (Ma et al., 2006). This function of SGK1 may relate to its ambivalent interaction with glutamate receptors as it enhances the excitatory effects of glutamate by upregulating AMPA receptors but at the same time enhances the expression of glutamate transporters that clear the synaptic cleft and terminate excitation (Benarroch, 2010; Lang et al., 2006). SGK1 is further associated with several neurological diseases such as Alzheimer (phosphorylation of the tau protein by SGK1), Parkinson (SGK1 upregulation coincides with the onset of dopaminergic cell death) and depressive disorders (Lang et al., 2006; Sakai et al., 2007; Sato et al., 2008). However, it is to date unclear if the role of SGK1 in brain pathology is deteriorative or supportive for neuronal function (Lang et al., 2010).

# Brain tumors

Today, cancer is one of the leading causes of death worldwide with a growing incidence, as the world's population statistically reaches higher ages. Among the many different groups of cancer, tumors of the brain are especially dangerous as the surgical resection of tissue and radiotherapy affect its function. Fast progression, infiltrative character and a tendency for relapse that is characteristic for many malignant brain tumor subtypes, account for a generally poor prognosis.

Despite huge efforts in the field of cancer biology and although the technical equipment and surgical techniques have improved dramatically, only little progress has been achieved in the therapy of brain tumors and for many brain malignancies, treatment remains only palliative.

- I. Two key questions in the field of brain tumor biology, which would significantly contribute to improved detection and therapy, remain unresolved: Identification of the exact cell of origin of brain tumor development.
- II. Elucidation of the cellular and molecular events that occur during brain tumor development.

## **Classification of primary brain tumors**

The terms "primary brain tumor" and "primary tumor of the central nervous system" refer to neoplasms of diverse origin, localization and histopathological appearance that develop from cells inside of the cranium. Brain tumors can be of benign or malign (cancerous) character. Their occurrence is typically life threatening as they increase the pressure in the cranium, thereby traumatizing the brain tissue. According to Fumari and colleagues, the incidence for primary brain tumors worldwide is seven per 100.000 individuals p.a., which equals about 2% of all primary tumors (Furnari et al., 2007).

The world health organization publishes a regularly updated classification system of the tumors of the CNS, conferring each tumor type a grade of malignancy (Table 2). This grading scheme (WHO I-IV) is based on histological observations and serves as a means to predict the progression of the brain tumor and to determine a suitable treatment. Grade I neoplasms include tumors with low proliferative potential and the possibility of cure after resection (e.g. pilocytic astrocytoma). Grade II lesions are infiltrative and may recur (e.g. diffuse astrocytoma). Grade III and IV tumors show histological evidence of malignancy including nuclear atypia and mitotic activity with grade IV neoplasms being necrosis-prone and recurring fast (e.g. anaplastic astrocytoma and glioblastoma (GBM), respectively) (Louis et al., 2007).

In the following, one astrocytic tumor, GBM, and two embryonic tumors, CNS primitive neuroectodermal tumor (CNS PNET) and atypical teratoid/rhabdoid tumor (AT/RT), all corresponding to WHO grade IV, will be described.

### *Glioblastoma multiforme*

GBM is the most frequent type of brain tumor, accounting for 12-15% of all intracranial neoplasms and 60-75% of astrocytic tumours (Ohgaki et al., 2005). It may manifest at any age but preferentially affects adults with a peak incidence between 45 and 75 years of age (Louis et al., 2007). In a population-based study (Canton of Zurich, Switzerland) the mean age of patients with GBM was 61.3 years with more than 80% of patients being older than 50 years whereas only 1% of patients were younger than 20 years (Ohgaki et al., 2004). GBM is characterized by its aggressiveness: hallmark features are uncontrolled cellular proliferation, diffuse infiltration, necrosis, robust angiogenesis, resistance to apoptosis and genomic instability (Furnari et al., 2007). The histopathology of GBM can be extremely variable. Generally, this tumor is an anaplastic, cellular glioma composed of poorly differentiated astrocytic tumor cells with nuclear atypia and mitotic activity. Microvasculature and/or necrosis are essential diagnostic features (Louis et al., 2007). A histological variant of GBM is giant cell glioblastoma that features bizarre, multinucleated cells and a high frequency of *TP53* mutations (Louis et al., 2007). Giant cell glioblastoma develops *de novo* and has a wider age distribution, including children.

GBM is subdivided in primary and secondary tumors. Primary GBM occurs *de novo* without previous malignancies and represents the majority of GBMs (about 90% of cases). This tumor appears typically in older patients (mean 62 years) (Ohgaki et al., 2004; Ohgaki et al., 2007). GBM may also develop from diffuse astrocytoma or anaplastic astrocytoma and is then termed secondary GBM. This tumor is less frequent (about 5% of cases) and typically appears in younger patients with a mean age of 45 years (Ohgaki et al., 2004; Ohgaki et al., 2005). In Children, high-grade gliomas account for 5-10% of intracranial neoplasms (Pollack, 1994).

### *Common genetic alterations in GBM*

The most frequent genetic alteration in both primary and secondary GBM is the loss of heterozygosity (LOH) 10q, either due to deletion of regions or loss of the entire chromosome 10 (Fujisawa et al., 2000; Fults et al., 1998; Ichimura et al., 1998; Rasheed et al., 1995). The region 10q comprises the *PTEN* sequence and is supposed to code for additional, not yet identified tumor suppressors (Ohgaki et al., 2007). *EGFR* is the most frequently amplified and over-expressed gene in GBM (Fuller et al., 1992) and *EGFR* amplification goes often along with structural alterations leading to constitutive activation of the receptor in a ligand-independent manner. Subsequently, cell proliferation is triggered by downstream induction of the PI3K (phospho-

inositol-3-kinase)/AKT and RAS pathway (Ciardiello et al., 2001). *PTEN* is mutated in 15-40% of cases and almost exclusively in primary GBM (Tohma et al., 1998). *TP53* mutations are crucial for the development of both primary and secondary GBM (65%/28% of cases). In the majority of precursor astrocytomas, these mutations are the first detectable genetic alterations (Ohgaki et al., 2007). Escape from TP53 (tumor protein 53) regulated proliferation control might also result from altered expression of the *MDM2* and *ARF* genes (Louis et al., 2007). Furthermore, alterations in the p16<sup>INK4a</sup>/RB1 pathway are frequently in both primary and secondary GBM (Nakamura et al., 2001). More recently, mutations in *IDH1*, coding for isocitrate dehydrogenase 1 that catalyzes the oxidative carboxylation of isocitrate to  $\alpha$ -ketoglutarat in the citric acid cycle, were identified in an analysis of protein-coding genes in GBM and subsequent studies demonstrated that these events are very frequent in secondary GBM (>70%) (Ohgaki et al., 2009; Parsons et al., 2008; Yan et al., 2009).

### *CNS PNET*

CNS PNET comprises a heterogeneous, rare group of embryonal tumors (3 to 7% of pediatric tumors) (Becker et al., 1983) that are predominantly found in children and adolescents, arise in the central hemispheres, brain stem or spinal cord and consist of un- or poorly differentiated neuroepithelial cells. The term CNS PNET is synonymous with the term supratentorial PNET that describes the extracerebellar localisation of this tumor in the CNS and distinguishes PNET from histologically similar but intratentorially occurring medulloblastomas. PNET consists of small cells with little cytoplasm (Dirks et al., 1996). A common feature of all tumor variants is early onset and aggressive clinical behavior (Louis et al., 2007).

CNS PNET shows a higher frequency of chromosome loss than gain, with loss of chromosome 4q in 50% of cases being the most common change (Nicholson et al., 1999). *N-MYC* amplifications, like the chromosome 2p24 amplification, occur frequently in CNS PNETs (Bayani et al., 2000; Behdad et al., 2010) and an increased TP53 immunoreactivity was found in human PNET samples, indicating an altered TP53 functionality or pathway (Eberhart et al., 2005). The majority of observed DNA copy number losses or chromosomal gains has not yet been associated to putative tumor suppressor or oncogenic loci (Li et al., 2005). Moreover, it has been shown that the dysregulation of major signalling pathways like sonic hedgehog (Shh), Wnt and Notch are involved in supratentorial PNET development (Taylor et al., 2000).

### *Atypical teratoid/rhabdoid tumor*

AT/RT is an embryonal tumor that manifests typically in children and accounts for 1.3% of pediatric brain tumors (Rickert et al., 2001). Originally, rhabdoid tumors were described in the kidney but can basically occur in any tissue. The first case of a



rhabdoid tumor in brain was reported 1985 (Montgomery et al., 1985). This tumor type was more precisely defined as "atypical teratoid/rhabdoid tumor" when occurring in the CNS to call attention to the disparate combination of rhabdoid, primitive neuroepithelial, epithelial and mesenchymal components and described as an entity in 1996 (Rorke et al., 1996).

The cellular morphology of AT/RT is described as rhabdoid (histologically similar to rhabdomyoblasts) with features like eccentrically placed nuclei, prominent eosinophilic nucleoli, abundant cytoplasm with eosinophilic globular inclusions and well-defined cell borders. The appearance of these cells can vary dramatically in tumors (Louis et al., 2007). Furthermore, cells with PNET characteristics and divergent differentiation along epithelial, mesenchymal, neuronal or glial lines representing a "teratoid" or abnormal combination, can occur (Louis et al., 2007). Due to this complex histopathological patterning, AT/RT is often misdiagnosed as medulloblastoma, PNET or choroid plexus carcinoma (Biegel et al., 2002). The character of AT/RT is highly aggressive, with reported average survival times after surgery ranging from 11 to 24 months (Burger et al., 1998; Chen et al., 2005; Hilden et al., 2004).

Rhabdoid brain tumors are most often characterized by loss of one copy of chromosome 22 (monosomy 22) and subsequent LOH for alleles on the remaining chromosome. Positioning strategies on chromosome 22 led to the identification of *hSNF5/INI1/BAF47/SMARCB1* as a rhabdoid tumor suppressor gene (Versteeg et al., 2002) and the loss of SMARCB1 has become a defining pathological feature for AT/RT. The SMARCB1 protein is part of the SWI/SNF ATP-dependent chromatin-remodelling complex (Schnitzler et al., 1998) and it regulates tumor suppression via the p16<sup>INK4A</sup>/Rb pathway. In 20-24% of tumors, homozygous deletions are detected while in other cases deletion of one allele occurs subsequent to a mutation of the other allele (Biegel, 1997; Tekautz et al., 2005). In context with the latter phenomenon, Sévenet and colleagues described a hereditary syndrome predisposing to rhabdoid tumors and to a variety of tumors of the CNS, which they termed "rhabdoid predisposition syndrome" (Sevenet et al., 1999). In addition, an altered TP53 functionality or pathway and MYC upregulation are typical for human AT/RT (Eberhart et al., 2005; Ma et al., 2010).

## The cell of origin in brain tumors

For a long time, glial cells were considered to be the brain tumor cells of origin since they show proliferation even in the adult brain. It was further anticipated that brain tumor cells of origin must show a phenotypical similarity to the respective tumor cells: for example, it was assumed that GBM was initiated by a glial cell that had undergone a process of dedifferentiation (Alcantara Llaguno et al., 2011). Advances in the field of stem cell biology during recent years have led to studies that support the notion of NSCs as cells of origin. Evidence has been found indicating that brain tumors arise from postnatal NSCs (B-cells) or the B-cell derived transit-amplifying precursors (C-cells) that exist in the postnatal brain in a germinal zone close to the lateral ventricles (Doetsch et al., 1999; Sanai et al., 2005) in which cells proliferate and generate neurons and glial cells. Two main features make stem cells prone to transformation: the capacity to self-renew and longevity, the latter allowing for an accumulation of mutations over long time periods that consequently may lead to oncogenesis (Visvader, 2011).

It is noteworthy that tumor initiation does not necessarily have to take place in a stem cell but may happen in a more differentiated precursor cell, which might be susceptible to mutations and still have or regain stem cell features. Doetsch and colleagues could show that neurogenic precursors became highly proliferative, retained stem cell qualities and showed properties of glioma cells when exposed to epidermal growth factor (EGF) (Doetsch et al., 2002). In the hematopoietic field, Krivtsov and colleagues could show that granulocyte macrophage progenitor cells (GMPs), which were turned into leukemic stem cells by over-expression of the fusion gene *MLL-AF9*, expressed only a small subset of the hematopoietic stem cell (HSC) gene set, were still more similar to GMPs and did not have to undergo a complex process of dedifferentiation (Krivtsov et al., 2006). The groups of Schuller and Yang demonstrated that upon the activation of Shh in NSCs of a mouse model for medulloblastoma, malignant transformation occurred in lineage-restricted granule cell progenitors (Schuller et al., 2008; Yang et al., 2008).

It is also important to note that a cell initially acquiring a mutation may not be the cell of origin but rather pass it on to progeny, which ultimately transforms. In this scenario, a mutated NSC acts as the cell of mutation whereas the more restricted progeny transforms and acts as the cell of origin (Liu et al., 2011).

Furthermore, the cell of origin concept must be distinguished from the concept of the cancer stem cell, which is defined as a cell within a tumor that possesses the capacity to self-renew and to generate the heterogeneous lineages of cancer cells that comprise the tumor (Clarke et al., 2006). The subset of cancer stem cells that sustains malignant growth and propagates the tumor is not necessarily related to the cell of origin (Visvader, 2011). This was demonstrated in the hematopoietic system by Jamieson and colleagues, who showed for chronic myelogenous leukemia (CML), a

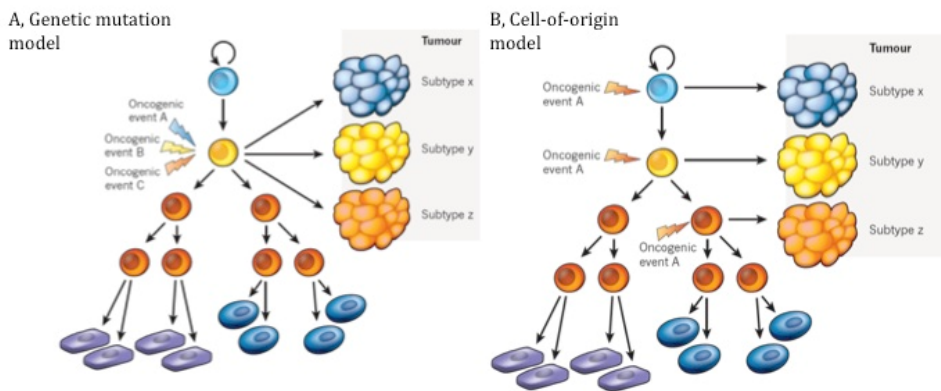
leukemia in which the HSC is the cell of origin, that granulocyte-macrophage progenitors acquire self-renewal capacity through a beta-catenin mutation and thereby constitute a cancer stem cell population (Jamieson et al., 2004).

**Table 2. WHO grading of primary tumors of the CNS.**

Tumors studied in this thesis are highlighted (table taken from Louis, 2007).

	I	II	III	IV		I	II	III	IV
<b>Astrocytic tumours</b>									
Subependymal giant cell astrocytoma	•				Central neurocytoma		•		
Pilocytic astrocytoma	•				Extraventricular neurocytoma		•		
Pilomyxoid astrocytoma		•			Cerebellar liponeurocytoma		•		
Diffuse astrocytoma		•			Paranglioma of the spinal cord	•			
Pleomorphic xanthoastrocytoma		•			Papillary glioneuronal tumour	•			
Anaplastic astrocytoma			•		Rosette-forming glioneuronal tumour of the fourth ventricle	•			
Glioblastoma				•	<b>Pineal tumours</b>				
Giant cell glioblastoma				•	Pineocytoma	•			
Gliosarcoma				•	Pineal parenchymal tumour of intermediate differentiation		•	•	
<b>Oligodendroglial tumours</b>					Pineoblastoma				•
Oligodendroglioma		•			Papillary tumour of the pineal region		•	•	
Anaplastic oligodendroglioma			•		<b>Embryonal tumours</b>				
<b>Oligoastrocytic tumours</b>					Medulloblastoma				•
Oligoastrocytoma		•			CNS primitive neuroectodermal tumour (PNET)				•
Anaplastic oligoastrocytoma			•		Atypical teratoid / rhabdoid tumour				•
<b>Ependymal tumours</b>					<b>Tumours of the cranial and paraspinal nerves</b>				
Subependymoma	•				Schwannoma	•			
Myxopapillary ependymoma	•				Neurofibroma	•			
Ependymoma		•			Perineurioma	•	•	•	
Anaplastic ependymoma			•		Malignant peripheral nerve sheath tumour (MPNST)		•	•	•
<b>Choroid plexus tumours</b>					<b>Meningeal tumours</b>				
Choroid plexus papilloma	•				Meningioma	•			
Atypical choroid plexus papilloma		•			Atypical meningioma		•		
Choroid plexus carcinoma			•		Anaplastic / malignant meningioma			•	
<b>Other neuroepithelial tumours</b>					Haemangiopericytoma		•		
Angiocentric glioma	•				Anaplastic haemangiopericytoma			•	
Chordoid glioma of the third ventricle		•			Haemangioblastoma	•			
<b>Neuronal and mixed neuronal-glial tumours</b>					<b>Tumours of the sellar region</b>				
Gangliocytoma	•				Craniopharyngioma	•			
Ganglioglioma	•				Granular cell tumour of the neurohypophysis		•		
Anaplastic ganglioglioma			•		Pituicytoma		•		
Desmoplastic infantile astrocytoma and ganglioglioma	•				Spindle cell oncocytoma of the adenohypophysis	•			
Dysembryoplastic neuroepithelial tumour	•								

A hallmark of cancer is its broad phenotypic variability: one tumor can comprise cells with different properties (intratumoral heterogeneity) (Visvader et al., 2008) and discrete tumor subtypes exhibiting differences in morphology and expression profile may develop within the same organ (intertumoral heterogeneity). While the concept of the cancer stem cell is suitable to explain intratumoral heterogeneity (Singh et al., 2003), two scenarios are conceivable that might lead to intertumoral heterogeneity: a) the genetic model, in which different mutations occur within the same target cell, resulting in different phenotypes and b) the cell of origin model in which different tumor subtypes arise from distinct cell lines of an organ (Figure 3). It is also conceivable that both cellular and molecular mechanisms interact (Visvader, 2011). Several studies have been performed to investigate the cellular origin of brain tumors. They applied two major experimental approaches: I) transgenic or conditionally targeted gene technologies to learn more about the effects of oncogenes and tumor suppressors in different spatial and temporal contexts and II) genetic alteration of cells and subsequent transplantation to study their respective tumor formation capacity (Visvader, 2011).



**Figure 3. Two models of intertumoral heterogeneity.**

A, genetic mutation model and B, cell-of-origin model (figure taken from Visvader, 2011).

Evidence supporting the NSC origin of brain tumors has come from several mouse genetic studies. Zhu et al. generated mice lacking expression of *Trp53* and a negative regulator of Ras, *Nf-1*, in mature astrocytes and type B lateral ventricle wall (LVW) cells (Zhu et al., 2005). Detectable growth of malignant astrocytomas was found to arise from the forebrain of the mice, to be in association with the LVW and to appear infiltrative. Furthermore, Zhu et al. found early loss of TRP53 (transformation

related protein 53) to be essential for the generation of astrocytomas in this specific mouse model. Based on their observations, the authors predicted a) the existence of a specific cell type(s) in the LVW which is more prone for transformation as compared to cells of other brain regions or b) the LVW as a favorable niche for tumor development (Zhu et al., 2005). The same group later showed that the generation of malignant astrocytomas under the conditions Zhu et al. used can be accelerated by introduction of an additional haploinsufficiency for *PTEN* (Kwon et al., 2008). Llaguno and colleagues performed a combined spatially and temporarily restricted *in vivo* gene targeting study using an inducible Cre-recombinase transgene under the control of the *Nestin* promoter and a stereotactic viral-mediated Cre-recombinase delivery to the LVW, respectively. All mice that were injected with virus into the LVW developed astrocytomas. Virus injection into the brain parenchyma did not result in tumor formation in case of four to eight weeks old adult mice while in one out of 12 injected, one to two days old post-natal mice tumor formation occurred. Moreover, the inducible approach showed tumor induction only for nestin-expressing neural stem/progenitor cells (Alcantara Llaguno et al., 2009). The study provides strong evidence that mutated neural stem/progenitor cells account for astrocytoma-initiating cells. However, no conclusion can be drawn if quiescent stem or proliferating progenitor cells (B- or C cells respectively) are the cells of origin. In a similar approach, Jaques and colleagues deleted certain combinations of tumor suppressor genes in LVW *GFAP* expressing cells *in vitro* and *in vivo* (Jacques et al., 2010). Jaques et al. could not only demonstrate that adult stem/progenitor cells of the LVW are brain tumor cells of origin, supporting earlier findings of Llaguno et al. and other groups, but also showed that the different combinations of mutations applied for cell transformation led to the development of two different tumor types.

The results of other studies point more toward transformed NSC-derived progeny like astrocytes or oligodendrocyte precursor cells (OPCs) as brain tumor cells of origin. Bachoo and colleagues studied the effect of a combined knockout of the *Cdkn2a* locus (*Ink4a/Arf<sup>f</sup>*) with an over-expression of permanently active epidermal growth factor receptor (*EGFR*) on astrocyte and neurosphere cell cultures. They found that both manipulated cell types generated glioblastomas after orthopic transplantation into mice and concluded that neural stem/progenitor cells as well as more differentiated cells can be transformed into cells of origin (Bachoo et al., 2002). However, the work is restricted by the fact that unphysiological cell culture effects and the presence of immature cell types in the cell cultures cannot be excluded (Visvader, 2011). Persson et al. found OPCs, which have a limited self-renewal capacity compared to NSCs, to be transformed by the expression of *v-erbB* (activated *EGFR*) under the control of the *S100 $\beta$*  promoter and could initiate oligodendroglioma formation upon transplantation of these cells into mice (Persson et al., 2010). Moreover, the group compared NSCs and OPCs as potential cells of origin in murine and human oligodendrogliomas applying gene expression profiling and found white matter OPCs to be closer related to oligodendroglioma cells and to

be more tumorigenic than SVZ NSCs. Based on results from human oligodendroglioma positioning with MRI technique, they suggested that these tumors arise from white matter cells rather than the lateral ventricles. The hypothesis of OPCs acting as oligodendroglioma-initiating cells was supported by a study of Sugiarto and colleagues who identified decreased NG2 asymmetry of OPCs in a mouse model as a defect in cell fate regulation that is associated with pre-malignant, abnormal self-renewal and neoplastic transformation of OPCs (Sugiarto et al., 2011). To further elucidate if NSCs or precursor cells function as brain tumor initiating cells, Liu et al. generated a mouse model for gliomagenesis by initiating sporadic *Trp53/Nf1* mutations in NSCs that could be tracked throughout the process of tumorigenesis (Liu et al., 2011). They observed an overexpansion and aberrant growth specifically in OPCs but not NSCs and confirmed the OPC nature of glioma cells. Furthermore, Liu and colleagues observed glioma formation upon introduction of *Trp53/Nf1* mutations in OPCs that were indistinguishable from those initiated from NSCs. The authors concluded with regard to their mouse model that NSCs might serve as cell of mutation while OPCs serve as the actual cell of origin.

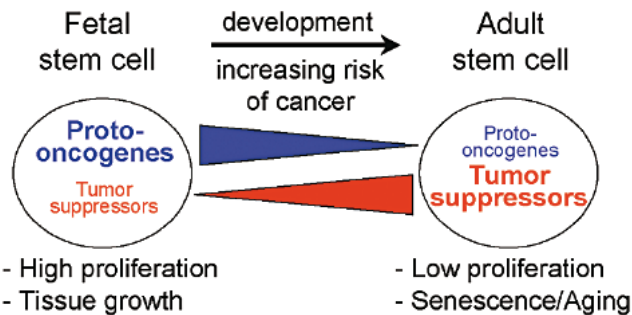
In conclusion, the studies mentioned here show an origin of murine brain tumors from both NSCs and more differentiated cells such as OPCs and indicate that different brain tumor types arise from different cells of origin. However, all these studies are based on animal models, and it remains to be elucidated if the findings also relate to human brain tumors. So far, indications for NSCs as cells of origin in human glioma development have been presented. Lim and colleagues developed an MRI-based classification of GBM according to their involvement of the SVZ and cortex and found that SVZ contact predicted for multifocality and recurrence at sites distant of the initial resection, suggesting a highly migratory and invasive cell of origin for this tumor type (Lim et al., 2007). Barami et al. reviewed 100 glioma patients and found that 93/100 patients' tumors involved the ventricular walls/SVZ (Barami et al., 2009). The observed tendency for high-grade gliomas to develop adjacent to the ventricles was further affirmed by Marsh et al. who reported an involvement of the NSC compartment in gross tumor volume in 103/104 cases (Marsh et al., 2012). However, the authors pointed out several restrictions of their studies, such as small patient numbers, analysis of adult patient material only and the merely observational character of their studies. In addition, it is impossible to monitor human glioma growth in real time. Therefore, further work is needed to verify these results and to fully investigate the cell of origin in human high-grade gliomas.

### **Genetic pathways in malignant brain tumors**

Since unlimited self-renewal is a hallmark of cancer and stem cells and cancer cells can be derived from self-renewing stem/progenitor cells, it is conceivable that these cell types utilize similar molecular mechanisms to maintain themselves.

Self-renewal mechanisms that allow stem cells to persist over a long period of time involve genetic pathways like Wnt, Shh and Notch that are potentially oncogenic (Pardal et al., 2005). Activation of these pathways in stem cells might render them prone to neoplastic transformation (Dahmane et al., 2001; Kanamori et al., 2007; Sareddy et al., 2009) as it may take fewer mutations to permanently activate such pathways that are absent in postmitotic cells. The risk of transformation, accompanying unlimited self-renewal, is reduced by tumor suppressor mechanisms that build a counterweight to active oncogenes and prevent neoplastic proliferation. In cancer cells, central tumor suppressor mechanisms are lost.

The balance of proto-oncogenes and tumor suppressor activity in stem cells changes throughout life (Figure 4) (Pardal et al., 2005). In fetal development, which is a short time of rapid stem cell proliferation to ensure the necessary tissue growth, the chance of acquiring mutations is relatively low and proto-oncogene activity dominates over tumor suppressor activity. In contrast, adult stem cells in a mature organism with developed tissues that need to be maintained are more quiescent and more tumor suppressor mechanisms are expressed to control the increasing risk of mutation-acquisition and transformation.



**Figure 4. Developmental changes in the balance between proto-oncogenes and tumor suppressors.**

(figure taken from Pardal, 2005)

### **Growth factor receptor signalling**

Growth factor receptors activate several signalling pathways involved in cellular growth and survival (Grzmil et al., 2010) and include EGFR, VEGFR (vascular endothelial growth factor receptor), PDGFR (platelet-derived growth factor receptor), HGFR (hepatocyte growth factor receptor) and others. Doetsch and colleagues showed the expression of *EGFR* in neural progenitor type C cells *in vitro* and

described that these cells become highly proliferative and invasive when exposed to EGF *in vivo*, indicating deregulated EGF receptor signalling as a first step toward transformation, enhancing self-renewal and proliferation (Doetsch et al., 2002). An altered cell signalling is very common in GBM and occurs e.g. after gene amplification. Truncating or exon-deleting mutations generate either a constitutively active form of a growth factor receptor or lead to ligand independence (Chakravarti et al., 2004; Maher et al., 2001; Sonabend et al., 2007). In different mouse models, EGFR activation in combination with knockout of certain tumor suppressors was shown to induce glioma formation (Bachoo et al., 2002; Bruggeman et al., 2007; Persson et al., 2010).

### **Oncogenes *Ras* and *Myc***

Normally expanding cells sense extracellular signals via growth factor receptor binding, leading to the activation of certain signalling pathways and to cell cycle activation and proliferation in a controlled manner. This dependence on extracellular signals and controlled proliferation activation is lost in tumor cells. Hyperactivation of receptor tyrosine kinases (RTKs) is a key feature of brain malignancies with the subgroup of growth factor receptors being particularly important in the development of gliomas (Alcantara Llaguno et al., 2011). Binding of the respective ligand to RTKs induces their dimerisation and autophosphorylation and triggers intracellular signal transduction cascades like the MAPK/RAS pathway. The GTPase RAS cycles between GTP-bound state (active) and GDP-bound state (inactive), mediated by GTPase-activating proteins and guanine exchange factors. Activated RAS induces the MAPK (microtubule-associated protein kinase) pathway, which leads to the expression of several transcription factors (e.g. *MYC*) and cellular components and ultimately to deregulation of proliferation and apoptosis, invasiveness as well as angiogenesis. High levels of active RAS have been reported for malignant astrocytomas, but only few *RAS* mutations have been found in tumor samples and it has been shown that RAS activation is induced upstream by alterations of RTKs like EGFR and PDGFR (Guha et al., 1997).

The *Myc* family of proto-oncogenes consists of *n-Myc*, *l-Myc*, *s-Myc* and *b-Myc*, with *n-Myc* and *c-Myc* being functionally equivalent (Shervington et al., 2006). *MYC* proteins comprise a helix-loop-helix leucine zipper, form heterodimers with the MAX protein and activate the transcription of hundreds of genes via binding to the E-box DNA sequence. *Myc* functions include the mediation of neuronal progenitor cell proliferation (Kenney et al., 2003; Oliver et al., 2003). Over-expression of *Myc* promotes malignant transformation and *c-MYC* and *n-MYC* are often found over-expressed in medulloblastomas (Pomeroy et al., 2002), GBM (Herms et al., 1999)



and PNET (Behdad et al., 2010). Myc induces the cell cycle via activation of *cdc25A* and down-regulates the synthesis of the CDK inhibitor proteins p15, p21 and p27 (Shervington et al., 2006). Moreover, Myc proteins have been found involved in the activation of tumor suppressors like *Trp53* and *Arf* and shown to induce apoptosis. Therefore, suppression of apoptosis, e.g. by deletion of *Trp53*, enables Myc proteins to acquire full oncogenic activity and allows for efficient neoplastic growth (Elson et al., 1995; Shervington et al., 2006). In addition, Myc proteins maintain an open state of chromatin, thus making cells susceptible for further genetic events (Fernandez et al., 2003).

### **Tumor suppressor genes**

For long time it was assumed that cancer is a result of mutations in proto-oncogenes that, when deregulated, together cause tumor formation (Stehelin et al., 1976). However, under the last decades the existence of genes that can suppress tumorigenicity has been proven and many such tumor suppressor genes have been identified. Tumor suppressors are divided into three categories: "caretakers", that prevent mutations by detecting and promoting the repair of DNA damage, "gatekeepers" that promote senescence or apoptosis upon mutation or cell stress and "landscapers" that regulate the microenvironment in which tumor cells grow (Macleod, 2000; Pardal et al., 2005). The functions of tumor suppressors are especially important for self-renewing, proliferating stem cells that may pass mutations to a large number of progeny, leading to the accumulation of mutations and possibly transformation. While caretaker tumor suppressors sustain normal stem cell activity, gatekeeper tumor suppressors block stem cell functions.

The nature of tumor suppressor genes is generally recessive as usually only one single functional gene is required for tumor inhibition. Charles J. Sherr defines three major features of classic tumor suppressor genes: 1) occurrence of biallelic inactivation in tumors, 2) acceleration of tumor susceptibility by a single mutant allele and 3) frequent inactivation of the same gene in sporadic cancers (Sherr, 2004). Inheritance of a single mutant allele dramatically increases the susceptibility to certain tumors and is the reason for familial cancer syndromes like Li-Fraumeni. Alternatively, silencing of a tumor suppressor gene can occur as a two-step process with LOH leading to cellular transformation. Furthermore, tumor suppressor genes like certain cyclin dependent kinase (CDK) inhibitors have been identified for which the inhibition of a single allele is sufficient to confer a growth advantage (Cook et al., 2000; Quon et al., 2001), a phenomenon referred to as haploinsufficiency. For many tumor suppressor genes, including *TP53*, *INK4A*, *ARF* and *PTEN*, haploinsufficient effects accelerating tumor progression have been observed when combined with collaborating mutations (Sherr, 2004).

*TP53* is assumed to be the most frequently inactivated gene in human cancers (Olivier et al., 2002) and The "Cancer genome atlas research network" stated mutations in this gene as a common event in primary GBM (Cancer genome atlas research network, 2008). The protein presents an important defense against tumor development as it is induced by and can counteract oncogene activation (Prives, 1998). *TP53* activates the expression of a broad range of genes, among them *Cdkn1a*, coding for p21<sup>WAF1/CIP1</sup> that binds and inhibits CDK2. In the unstressed cell, *TP53* is kept inactive and marked for degradation by the protein MDM2. Upon cellular noxes like DNA damage or hypoxia, this complex dissociates and *TP53* triggers a global transcriptional response that includes proliferation inhibition and/or induction of apoptosis.

*TRP53* has been shown to suppress the self-renewal function in adult murine neural stem/progenitor cells (Meletis et al., 2006) and it has been demonstrated that its inactivation, together with other genetic events, promotes a highly self-renewing and transformation-prone state of murine NSCs (Zheng et al., 2008).

The *TP53* pathway is regulated by the *CDKN2A* locus that encodes two alternative transcripts, *INK4A* and *ARF*. While p16<sup>INK4A</sup> blocks the ability of CDK4 and CDK6 to phosphorylate RB proteins, p19<sup>ARF</sup> protects *TP53* from MDM2. p16<sup>INK4A</sup> and p19<sup>ARF</sup> inhibit the self-renewal capacity of several types of stem cells. Two of the major mechanisms by which BMI1 promotes neural stem cell self-renewal are the inhibition of *INK4A* and *ARF* (Pardal et al., 2005). Oncogene induction, e.g. over-active RAS, is sensed by the *INK4A-ARF* locus that triggers the cancer protective *TP53*/RB pathways (Serrano et al., 1997). Because of its key position in tumor suppression, *INK4A* and *ARF* are targets of cancer deregulation and deletion of the *CDKN2A* locus occurs frequently in GBM (Cancer Genome Atlas Research Network, 2008). In this context, it remains enigmatic why two genes that regulate such highly important suppressor pathways were evolutionary organized at the same genetic locus (Sherr, 2004).

### **Effects of endoplasmic reticulum stress in cancer cells**

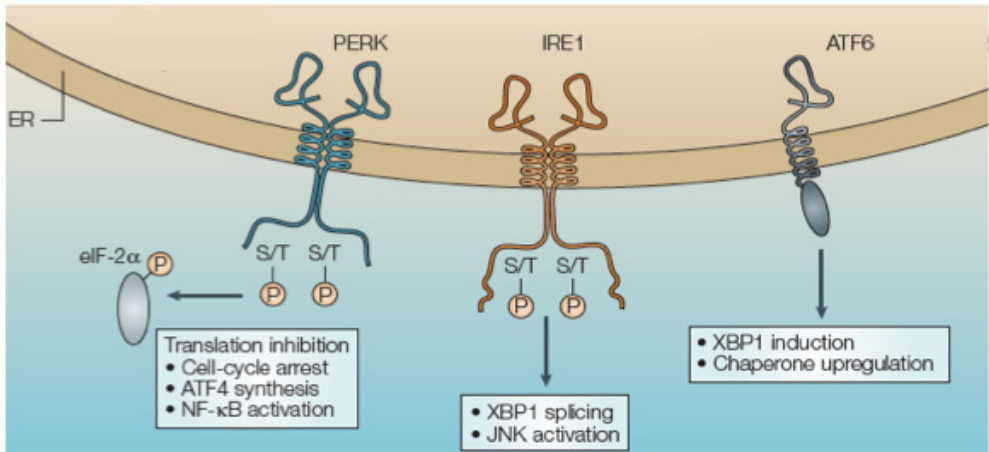
The endoplasmic reticulum (ER) of eukaryotic cells is a compartment in which nascent proteins are correctly folded and modified, a process orchestrated by a variety of molecular chaperones and folding enzymes that prevent aggregation of proteins. Malformed proteins are retranslocated to the cytosol and degraded via the proteasome.

Environmental stress conditions like hypoxia, nutrient deprivation (glucose, amino acids) and pH changes can affect the ER and disrupt a proper protein folding and maturation. As a consequence, a signal-transduction cascade termed the unfolded protein response (UPR) is triggered to prevent accumulation of unfolded proteins (Ma et al., 2004). The UPR is capable of balancing pro-survival signals via NF- $\kappa$ B

activation (Jiang et al., 2003) and the initiation of apoptotic pathways (Nakagawa et al., 2000). While it is the primary effect of the UPR to protect the ER and the cell, it leads to cell death in cases of prolonged stress. The pro-apoptotic role of the UPR might be exploited therapeutically: Lee and colleagues treated tumor cells facing ER stress with proteasome inhibitors *in vitro*, thus preventing the degradation of unfolded proteins and inducing the accumulation of ER-associated degradation substrates (Lee et al., 2003) and could in this way push the UPR toward induction of apoptosis and tumor cell death.

There are three branches of the UPR (eIF2alpha/IRE1/ATF6) with the ability to sense the folding capacity of the ER (Figure 5) (Wiseman et al., 2005). While the IRE1 and ATF6 pathways primarily increase the folding capacity of the ER by upregulating ER chaperones and folding enzymes (Calfon et al., 2002; Yoshida et al., 2001), phosphorylation of eIF2alpha by PKR-like ER kinase (PERK) and three other kinases (GCN2/PKR/HRI) induces translation inhibition, thereby reducing the amount of protein in a cell (Harding et al., 1999; Shi et al., 1998), loss of cyclin D1 and G1 cell cycle arrest (Brewer et al., 2000). Upon stress relief, the translational block is reversed by dephosphorylation of eIF2alpha, which is performed via a complex containing the enzyme phosphatase PP1 and its essential non-enzymatic cofactor GADD34 (Wiseman et al., 2005). SMARCB1 has been shown to bind PP1/GADD34 in solution and to stimulate its phosphatase activity (Wu et al., 2002).

Cancer cells of fast growing tumors with inadequate vascularization are likely to encounter a range of adverse physiological conditions like hypoxia and nutrient starvation that contribute to ER stress. Indeed, several studies report the activation the UPR in solid tumors. Target genes of the UPR have been shown upregulated in primary cells from breast tumors (Fernandez et al., 2000), hepatocellular carcinomas (Shuda et al., 2003), gastric tumors (Song et al., 2001) as well as oesophageal carcinomas (Chen et al., 2002) and an induction of the UPR has been demonstrated for *Myc*-amplified neuroblastoma cells (Yaari-Stark et al., 2010) and *HRAS*-amplified human melanocytes (Denoyelle et al., 2006).



**Figure 5. Components of the mammalian unfolded protein response.**

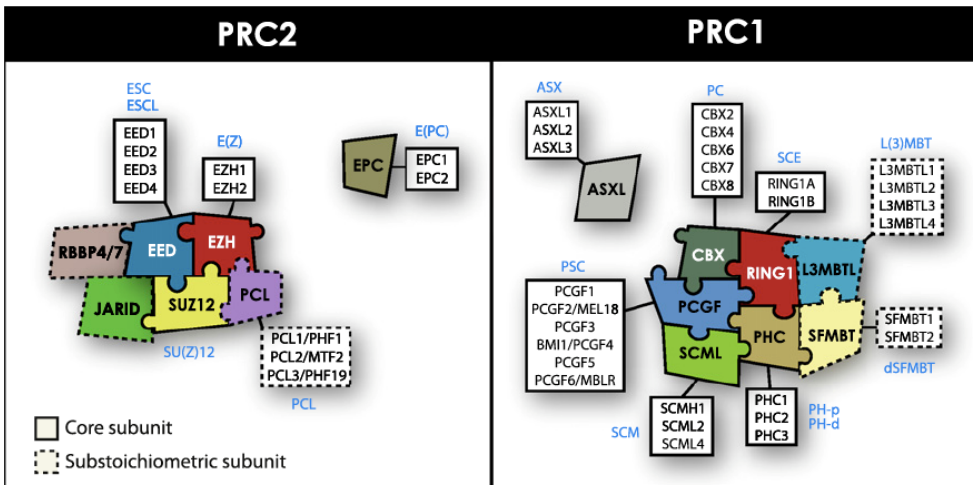
A transient inhibition of the protein synthesis during the UPR is achieved via PERK-mediated phosphorylation of eIF2alpha. PERK also activates NF-κB and induces ATF4 synthesis. ATF6 upregulates ER chaperones and induces *XBP1* transcription, which is then spliced by the endonuclease of activated IRE1, resulting in the synthesis of a more potent transcription factor than is encoded by the unspliced transcript (S/T-P: serin/threonin phosphorylation; figure adapted from Ma, 2004).

### **Polycomb group complex mediated gene regulation**

Polycomb Group (PcG) proteins were discovered in *Drosophila melanogaster* as the products of genes that are required to prevent inappropriate expression of homeotic (Hox) genes (Schwartz et al., 2007). Together with the activating Trithorax proteins, they were found to regulate the correct developmental patterning of *Drosophila*. Trithorax Group (TrxG) and PcG complexes were classically assumed to form a binary epigenetic switch that dictates a functionally inactive or active state of the target gene (Schwartz et al., 2008). Studies with mice and other model organisms revealed new PcG family members and indicated a more complex PcG protein machinery in mammals compared to *Drosophila*. While expressed in all multicellular model species and consistently involved in developmental patterning (Sparmann et al., 2006), the number of PcG genes expanded during evolution from approximately 15 in *Drosophila* to 37 in mouse and human (Sauvageau et al., 2010). While the role of PcG complexes in body segment formation is conserved in vertebrates and Polycomb mutants exhibit skeletal malformations (van der Lugt et al., 1994), in mammals, PcG complexes are also associated with embryonic and adult stem cell maintenance as well as neoplastic development.

The Polycomb group comprises a diverse set of proteins that assemble in chromatin-associated complexes (Figure 6) (Sauvageau et al., 2010). Gene duplications that occurred during evolution resulted in paralogous subunits that diverged in their structural domains. In mammals, two main Polycomb repressive complexes (PRCs) have been identified whose composition is context-dependent. The core of PRC1 consists of one subunit of the PCGF, CBX, PHC, SCML and RING1 (contains a monoubiquitylation E3 ligase) paralog groups. The core of PRC2 comprises SUZ12, an EED isoform, EZH1 or EZH2 (catalyze trimethylation), PCL (enhances methyltransferase activity), RBBP4/7 and JARID (help to recruit and modulate the enzymatic activity of PRC2) (Sauvageau et al., 2010).

PcG complexes are targeted to specific loci and repress gene expression through modification of histone tails and compaction of chromatin. In *Drosophila*, certain DNA sequences called Polycomb response elements (PREs) represent marks that are recognized by specific recruitment factors (PHO and PHO-like proteins, (Wang et al., 2004)) that target the PRCs to these sites. Although it was recently shown that similar to invertebrates, PREs may act as entry sites for PcGs in vertebrates, too (Sing et al., 2009), the binding sites of Polycomb proteins in mammals remain to be elucidated.



**Figure 6. Diversity of PRC1 and PRC2 complexes formed by vertebrate PcG proteins.** Subunits and paralogs of the complexes are indicated; the *Drosophila* homolog of each subunit is indicated in light blue. Contacts do not represent the actual interactions (figure taken from Sauvageau, 2010).

In a highly simplified model, Polycomb transcriptional silencing starts with the binding of PRC2 initiation complex to the respective target gene and methylation of histone proteins via EZH2, primarily at lysine 27 of histone H3 (H3K27). PRC1 is able to recognize the trimethylation mark of H3K27 (H3K27me<sup>3</sup>) through the chromodomain of PC, neighboring nucleosomes are brought into the proximity of PRC2 and methylation is spread over extended chromosomal regions (Schwartz et al., 2006; Sparmann et al., 2006). Transcriptional repression might then occur via direct inhibition of the transcriptional machinery, ubiquitylation of histone 2A lysine 119 (H2AK119), chromatin compaction as well as through recruitment of DNA methyltransferases (DNMTs) (Cao et al., 2005; Dellino et al., 2004; Francis et al., 2004; Sparmann et al., 2006; Wang et al., 2004).

Several studies that investigated mouse or human embryonic stem (ES) cells report the direct, PcG-mediated repression of developmental regulators that must be suppressed to maintain pluripotency and plasticity of ES cells (Boyer et al., 2006; Lee et al., 2006). Because PcGs prevent stem cells from undergoing differentiation and keep signalling pathways active, deregulation of PcG proteins can misguide a normal stem cell toward a malignant state. PcG proteins are found over-expressed in a broad variety of tumor types (reviewed in (Sauvageau et al., 2010; Sparmann et al., 2006)) and PcG target genes are enriched among the group of hypermethylated genes in ovarian cancer, follicular lymphoma and GBM (Martinez et al., 2009; O'Riain et al., 2009; Teschendorff et al., 2010), driving expression changes associated with carcinogenesis. Sauvageau and colleagues suggest that the DNA methylation mechanism of PcG complexes locks cells in an undifferentiated state, predisposing them to malignant transformation (Sauvageau et al., 2010).

The great diversification of PcG genes that has taken place in vertebrates generated paralogous subunits that can substitute each other but possess different catalytic activities. Changing components of a PRC complex influence its activity dramatically. In other words, while over-expression of a PcG protein like BMI1 could render a cell malignant or pre-malignant, dominance of its paralog PCGF2/MEL18 might function in an antagonistic way, protecting the physiologic state of the cell (Sparmann et al., 2006).

## **Discovery of BMI1**

BMI1 (B cell-specific Mo-MLV integration site 1) was discovered in 1991 by two research groups that followed the same approach to identify novel genes cooperating with *c-myc* (Haupt et al., 1991; van Lohuizen et al., 1991). The researchers independently performed a retroviral insertional mutagenesis study with E $\mu$ -*myc* mice that possess *c-myc* under an immunoglobulin heavy chain enhancer (Adams et al., 1985). They used Moloney murine leukemia virus (Mo-MLV) that promotes

tumorigenesis by inserting near or within cellular oncogenes that are consequently over-expressed or structurally altered (Harris et al., 1988; Schmidt et al., 1988). Leukemia virus infection accelerated the development of B lymphoid tumors in the mice and in 50% of cases a provirus was contained within the *Bmi1* locus. Not much later, the sequence similarity of BMI1 to the Drosophila PSC protein was discovered (Brunk et al., 1991; van Lohuizen et al., 1991) and in 1997, evidence for the existence of a mammalian Polycomb complex including BMI1 was presented (Alkema et al., 1997).

### **The Janus face of BMI1: functions in development, stem cell self-renewal and cancer**

Insight about the role of BMI1 during development was gained with the help of a transgenic mouse model in which *Bmi1* had been knocked out (van der Lugt et al., 1994). *Bmi1*-null mice displayed strong phenotypical alterations such as decrease in the number of hematopoietic cells, neurological abnormalities (e.g. ataxic gait, seizures) and posterior transformation; the latter observation confirming a BMI1 effect on a number of Hox genes and being in line with the role of the BMI1 homologue *Psc* in *Drosophila*.

These findings indicated the importance of BMI1 in morphogenesis during embryonic development and a role of BMI1 in hematopoietic stem cell regulation throughout pre- and postnatal life (van der Lugt et al., 1994). Park and colleagues could further show that *Bmi1* is expressed in adult and fetal murine and adult human HSCs, that HSCs are significantly reduced in postnatal *Bmi1*-null mice and that transplantation of fetal liver and bone marrow cells from these mice fails to permanently re-establish a self-renewing HSC population in irradiated mice (Park et al., 2003). Based on the facts that hematopoiesis is disturbed in *Bmi1* knockout mice, that *Bmi1* expression is restricted to primitive bone marrow cells in human and mouse and that it is expressed in myeloid leukaemia, Lessard and Sauvageau anticipated *Bmi1* to be a regulator gene of both normal and leukemic HSCs (Lessard et al., 2003). In a serial transplantation study they demonstrated that *Bmi1*<sup>-/-</sup> leukemic bone marrow cells could not repopulate secondary recipients: none of the transplanted mice developed acute myeloid leukaemia. While highly proliferative leukemic blasts were either not or only weakly tumorigenic, retroviral over-expression of *Bmi1* rescued those cells and rendered them highly tumorigenic, pointing toward the establishment of stem cell features by BMI1 (Lessard et al., 2003).

Molofsky and colleagues demonstrated that *Bmi1* deficiency also affects self-renewal of embryonic and adult NSCs. In a neurosphere assay with E14.5 CNS stem cells and P0 and P30 SVZ adult stem cells they found that *Bmi1* knockout reduced the ability to form spheres *in vitro*. This reduction of self-renewal was strongest in P30 cultures

and increased over time, indicating a depletion of adult NSCs upon loss of *Bmi1*. At the same time, *Bmi1* deficient, restricted neuronal and glial progenitors were shown to proliferate normally in culture (Molofsky et al., 2003). In another study that focused on the cerebellum of embryonic and adult *Bmi1* deficient mice, phenotypical changes like overall reduced size of the cerebellum as well as a reduction in proliferation and increased apoptosis in the external granular layer were reported (Leung et al., 2004). In the same study, BMI1 was found over-expressed in different medulloblastoma cell lines, pointing toward a role for *Bmi1* in neural development as well as in brain tumor formation. A following study complemented these findings and showed that medulloblastoma formation in *Bmi1*-null mice is initiated but further tumor growth is blocked. The final extinction of the remaining tumor cells indicated the BMI1-requirement for medulloblastoma maintenance (Michael et al., 2008).

### **The *Cdkn2a* locus as a classical BMI1 target**

Experiments with mouse embryonic fibroblasts (MEFs) derived from *Bmi1*<sup>-/-</sup> mice revealed that these cells had a significantly reduced proliferation rate and showed premature entry into senescence compared to wild-type cells while *Bmi1* over-expression induced immortalization (Jacobs et al., 1999). To elucidate the underlying molecular mechanisms, Jacobs et al. used MEF cell material to analyze the transcription- and translation level of proteins involved in cell cycle regulation. They found *Ink4a* and *Arf* to be upregulated in *Bmi1*<sup>-/-</sup> MEFs and observed a dramatic rescue of the typical cerebellar defects in *Bmi1*<sup>-/-</sup> mice when those animals were crossed with *Ink4a*-null mice (Jacobs et al., 1999). In subsequent experiments, the *Cdkn2a* locus, encoding both tumor suppressors, was shown to be directly BMI1-regulated (Bracken et al., 2007). The finding that *Ink4a* and *Arf* represent main BMI1 targets also helped to explain the collaboration of BMI1 with potent oncogenes like RAS and MYC in cell transformation and tumor induction (Datta et al., 2007; Haupt et al., 1991; Hoenerhoff et al., 2009; Jacobs et al., 1999; Jacobs et al., 1999; van Lohuizen et al., 1991) as *Ras* or *Myc* over-expression triggers a cellular anti-oncogenic, apoptosis and/or senescence-inducing response via *Ink4a-Arf* (Prendergast, 1999; Serrano et al., 1997).

### **Additional functions of BMI1**

Two studies published by the groups of Sean Morrison and Maarten van Lohuizen in 2005 manifested the central role of BMI1-mediated *Cdkn2a* repression in NSC self-renewal but indicated at the same time the existence of alternative BMI1 target genes. Neurosphere cultures derived from LVW tissue of adult mice that showed dramatically reduced self-renewal and proliferation upon *Bmi1* knockout could be



partially rescued upon either *Ink4a* or *Arf* deletion and almost be totally rescued upon deletion of both genes (Bruggeman et al., 2005; Molofsky et al., 2005). However, *Ink4a-Arf* deficiency in *Bmi1*-null mice could not completely restore the cerebellar development of wild-type littermates and did not prevent the death of those mice (Molofsky et al., 2005). In a subsequent study, Bruggeman et al. applied a mouse model for GBM that features deficiency for *Ink4a* and *Arf* in combination with a constitutively active EGFR (Bachoo et al., 2002). They found that BMI1 must also regulate *Ink4a/Arf* independent pathways since tumors appeared later, had a different histological grading and underwent gene expression changes to compensate for the BMI1-absence. In an shRNA-based *Bmi1* knockdown approach in neural progenitor cells, *Ink4a/Arf* expression levels were found unaltered after acute reduction of BMI1 while *Cdkn1a*, coding for p21<sup>CIP</sup>, a cell cycle inhibitor that maintains a stable Rb-E2F complex, was rapidly upregulated upon knockdown induction (Fasano et al., 2007). Reduced neurosphere frequency and size as well as decreased cell proliferation and survival could be rescued by knockdown of *Cdkn1a*, indicating that BMI1 functions as a *Cdkn1a* repressor (Fasano et al., 2007). More recently, Fasano and colleagues reported an increase in neurosphere self-renewal upon *Bmi1* over-expression that is accompanied by higher levels of the transcription factor FOXG1 and suggested the cooperation of BMI1 and FOXG1 in NSC self-renewal promotion (Fasano et al., 2009).

Liu and colleagues published a study that links BMI1 to the tumorsuppressive DNA damage response (DDR) that has been shown to play a role in mediation of cellular senescence, growth arrest and cell death and that can be limiting for self-renewal of cancer stem cells when induced by oncogenes (Viale et al., 2009; Zhou et al., 2000). Liu et al. performed experiments with *Bmi1*<sup>-/-</sup> thymocytes and found elevated levels of reactive oxygen (ROS) species that were not accompanied by changes in *Ink4a/Arf* transcription. ROS impaired mitochondrial respiration function that damaged DNA and induced cellular DDR *in vitro*. Moreover, treatment of *Bmi1* knockout mice with antioxidants or interruption of the DDR could rescue some phenotypical aspects *in vivo* (Liu et al., 2009). Concluding their results, the authors suggested a role for BMI1 in maintaining the redox homeostasis in stem cells.

## **The role of BMI1 in brain tumor biology**

*BMI1* has been found over-expressed in neuroblastomas and the majority of human medulloblastomas and is suggested to promote initiation, development and maintenance of neuroblastomas and development, maintenance but not initiation of medulloblastomas, respectively (Cui et al., 2007; Leung et al., 2004; Michael et al., 2008). BMI1 has also been found over-expressed in GBM (Abdouh et al., 2009) and it has been shown that BMI1-deficiency in murine NSCs impedes tumor growth and leads to the development of a less malignant phenotype *in vivo* (Bruggeman et al.,

2007). Furthermore, BMI1 was found over-expressed in GBM cells carrying the stem cell marker CD133 *in vitro* and to prevent apoptosis and/or differentiation in this population. Viral knockdown of *Bmi1* in GBM cells that were subsequently transplanted intracranially into mice hampered tumor development and prevented the death of the animals. Analysis of the significantly smaller *Bmi1* knockdown tumors revealed massive apoptosis, indicating the requirement of BMI1 for tumor development (Abdouh et al., 2009). However, the role of BMI1 in tumor initiation had to be reconsidered when He and colleagues presented studies featuring a mouse model that expressed *Bmi1* under the Nestin promoter. No effect on the proliferative potential of the LVW stem cell pool was detected in two to four months old mice and no brain malignancies developed in these animals. Based on the observed discrepancy between *in vitro* and *in vivo* results, He et al. assumed a more profound BMI1 effect in cultured cells because of higher levels of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> due to unphysiological culture environment. While they considered a primary role of BMI1 in tumor formation as unlikely, He and colleagues rather emphasized a supportive function of BMI1 for other strong, *Ink4a/Arf* inducing oncogenes (He et al., 2009). In a recent study applying mice with a mild enhancement of *Bmi1* expression in embryonic and postnatal neural stem/progenitor cells, increased proliferation in the LVW in contrast to He et al. was observed. The inability of *Bmi1* over-expression alone to induce tumor formation, however, was confirmed, thus underlining the role of BMI1 as a facilitator of transforming events (Yadirgi et al., 2011).

In conclusion, the hitherto published data indicate different BMI1 roles that are relevant in the context of brain tumor biology, namely an involvement in the self-renewal of normal NSCs and, depending on the studied tumor type, an association to initiation, development and maintenance of brain tumors. The question if increased BMI1 expression in NSCs leads to transformation and brain tumor initiation or rather serves to pave the way for other oncogenes, persists. Moreover, only few BMI1 target genes have been identified so far and it remains to be resolved how BMI1 exerts its functions in an *Ink4a/Arf*-repression independent manner.



# AIMS OF THIS THESIS

## Paper I:

Investigate if a pharmacological intervention with the glucocorticoid system influences the lifespan and symptoms of a Rett syndrome mouse model.

## Paper II:

Identify genetic events that induce brain tumor formation from neural stem/progenitor cells.

## Paper III:

Investigate cellular effects of *Bmi1* over-expression in neural stem/progenitor cells and identify novel direct BMI1 target genes.



# SUMMARY OF PAPERS

## Paper 1

Pharmacological interference with the glucocorticoid system influences symptoms and lifespan in a mouse model of Rett syndrome.

### Rationale

The majority of RTT cases are caused by mutations in the X-linked gene *MECP2* that acts as a transcriptional regulator. Although the gene was identified already in 1999, it still remains unclear how the dysfunction of MeCP2 leads to the different symptoms characteristic for RTT. A fundamental question in the field is which of the genes that are dysregulated in the absence of MeCP2 are directly implicated in the RTT phenotype. Nuber et al. previously showed that the glucocorticoid-mediated genes *Sgk1* and *Fkbp5* are upregulated in brain tissue of *Mecp2*-null mice when compared to wild-type animals already before the onset of symptoms and that MeCP2 binds to different genomic sites of these genes (Nuber et al., 2005). These findings indicate that MeCP2 acts as a negative modulator of glucocorticoid gene expression. Based on these results, in paper one we investigated whether a pharmacological activation or inhibition of the glucocorticoid system can aggravate or attenuate symptoms of an RTT mouse model.

### Results

We orally treated *Mecp2*-null male mice (*Mecp2<sup>0/y</sup>*), which show a rapid disease progression, and *Mecp2* heterozygous female mice (*Mecp2<sup>+/-</sup>*), which have a milder phenotype and a later onset of symptoms, with the rodent glucocorticoid corticosterone and the glucocorticoid receptor inhibitor RU486. We also included wild-type male and female mice in our study, which allowed us to distinguish RTT-related symptoms from other effects related to corticosterone or RU486. Our investigations revealed that the activation of the glucocorticoid system by oral application of corticosterone has a negative impact on the applied RTT mouse model. A low and a high dose corticosterone treatment resulted in a significantly shorter lifespan of male *Mecp2<sup>0/y</sup>* mice. Female *Mecp2* heterozygous mice are known to reach a

normal age and we did not observe any difference in lifespan for the mutant, corticosterone-treated animals compared to the vehicle treated control group. However, female *Mecp2*<sup>+/-</sup> mice receiving corticosterone showed a significantly earlier onset of certain motor symptoms such as hindlimb claspings and tremor. While no effect on the lifespan of RU486 treated *Mecp2*-null mice was observed, application of RU486 prolonged the hindlimb claspings-free lifetime of *Mecp2*<sup>ly</sup> males and the hindlimb claspings- and hypoactivity-free lifetime of *Mecp2*<sup>+/-</sup> females. Further analysis of the motor function and performance of *Mecp2*-null mice applying rotarod and open field tests showed a disturbed motor function of the mutant mice compared to wild-type animals. RU486 treated *Mecp2*-null mice performed significantly better than controls in the rotarod test and showed a trend toward increased travelled distance, while corticosterone treatment led to a significantly inferior rotarod performance as compared to RU486-treated animals and a trend toward decreased travelled distance. qPCR analyses of total brain RNA isolated from drug-treated, female mutant mice confirmed that the application of corticosterone and RU486 correlates with an up- and downregulation of the MeCP2 target genes *Fkbp5* and *Sgk1* in *Mecp2*<sup>+/-</sup> mice as compared to vehicle-treated animals.

## Conclusion

In summary, our investigations revealed that the activation of the glucocorticoid system by oral application of corticosterone has a negative impact on a RTT mouse model. We observed a significantly reduced lifespan of hemizygous male *Mecp2*<sup>ly</sup> mice, and a significantly earlier onset of symptoms in heterozygous female *Mecp2*<sup>+/-</sup> mice. The opposite effect, a prolonged symptom-free lifetime of *Mecp2* mutant female and male mice was achieved by treatment with the glucocorticoid antagonist RU486. Furthermore, RU486 application improved the motor function of male *Mecp2* mutant mice. Together with previous findings of an overactivation of glucocorticoid target genes in *Mecp2* mutant mice already before symptom onset (Nuber et al., 2005), the response to pharmacological manipulation of the glucocorticoid system in the present study indicates that this hormone system contributes to motor symptoms in a RTT mouse model. To date, RTT patients are generally treated symptomatically. One approach toward a specific therapy might be to pharmacologically target factors that are downstream of MeCP2 and whose gene expression is altered in RTT. The data we present here indicate that the inhibition of the glucocorticoid hormone system might represent a novel therapeutic avenue for this disease.

## Perspective

Based on the finding that an altered glucocorticoid hormone system contributes to the RTT phenotype, future studies should focus on the exact pathomechanism that links glucocorticoids to RTT symptoms. In this context, central questions that remain to be elucidated are

1. Which brain regions of *Mecp2* mutant mice are primarily affected by the upregulation of glucocorticoid-inducible genes and to which extent can the expression of these genes in these specific regions be influenced by pharmacological intervention?
2. Two receptor types mediate the regulation of glucocorticoid effects, the GR and the MR. Which role do these two receptors play in development of RTT symptoms? Since certain RTT-like symptoms of *Mecp2*<sup>+/-</sup> mice could be improved by blocking the GR receptor with an RU486 treatment, can this effect be increased by targeting GR/MR in combination?
3. Does the activation or inhibition of the glucocorticoid hormone system lead to detectable histological changes in certain brain areas of *Mecp2* mutant mice such as hippocampus, cortex and basal ganglia, which could explain the observed influence on RTT-specific symptoms?

In addition to these questions that can be approached experimentally, the fact that glucocorticoid treatment of *Mecp2* mutant mice had a negative effect on the development of RTT-like symptoms demands a critical evaluation of these drugs as a medication for RTT patients.



## Paper 2

Definition of genetic events directing the development of distinct types of brain tumors from postnatal neural stem/progenitor cells.

### Rationale

Brain tumor types are generally classified based on their histopathological features and named after the normal cell type they resemble best (e.g. astrocytoma, ependymoma or oligodendroglioma). However, little is known about the exact cellular origin of brain tumors. To which extent a tumor phenotype is determined by the cell it originates from or by the genetic events that take place during tumorigenesis remains to be elucidated.

In the first part of paper two, we asked a) which genetic alterations transform neural stem/progenitor cells into brain tumor generating cells and b) if the respective tumor subtype is stable or if it can be converted into another one by further genetic manipulation. To address these questions, we over-expressed different combinations of five candidate genes -*MYC*, *HRAS (V12)*, *Bmi1*, *Ezh2* and *FoxM1*- in *Trp53* deficient and wild-type neural stem/progenitor cells and orthotopically transplanted these cells into the brain of syngeneic mice.

A detailed gene expression analysis revealed the specific expression of a set of genes that are related to ER stress in the generated AT/RT-like cells. Since human AT/RTs which lack SMARCB1 and murine fibroblasts deficient for *Smarcb1* also show high expression of ER stress pathway genes, we investigated the role of ER stress and the functional relation to SMARCB1 in the second part of paper two.

### Results

To identify genetic combinations that lead to tumor formation, we transduced wild-type and *Trp53*<sup>-/-</sup> neural stem/progenitor cells with retroviral over-expression vectors coding for *MYC*, *HRAS (V12)*, *Bmi1*, *Ezh2* and *FoxM1* and transplanted these cells intracranially into mice. We found that a knockout of *Trp53* together with the over-expression of certain oncogenes is necessary for cell transformation as no brain tumors developed from wild-type, empty vector wild-type or empty vector *Trp53*<sup>-/-</sup> cells. Moreover, we found that the over-expression of either *MYC* or *HRAS* in *Trp53*<sup>-/-</sup> cells was sufficient for tumorigenesis while the over-expression of *Bmi1*, *Ezh2* and *FoxM1* alone was insufficient to generate tumors. Histological analysis of the induced tumors revealed the formation of three distinct subtypes, namely a high-grade glioma upon *HRAS* over-expression, CNS PNET upon *MYC* over-expression and an AT/RT-like

tumor upon over-expression of a combination of *HRAS* and *MYC*. The histological classification was corroborated by a gene set enrichment analysis (GSEA). Cells isolated from these tumors showed a high sphere formation rate *in vitro* and a high frequency of cells with tumor-forming capacity *in vivo* when used for serial transplantations.

To test the stability of the induced brain tumor types, sequential oncogene over-expression experiments were performed. We FACS-isolated tumor cells from gliomas or PNETs and transduced those cells with either *MYC*- or *HRAS*-over-expression vectors respectively and retransplanted these cells. Histological and gene expression analysis showed that while over-expression of *MYC* in *HRAS*-established gliomas did not change the tumor phenotype, over-expression of *HRAS* could convert *MYC* over-expressing PNETs into AT/RT-like tumors.

To gain insight about the molecular characteristics of the AT/RT-like tumor type, we searched for common transcription factor binding sites upstream of the genes more highly expressed in the brain tumor subtype. We found an over-representation of a motif called NFE2, which is bound by NRF2 and NF-kB. These transcription factors are associated with the induction of certain molecular pathways that are activated as a response to ER stress and are part of the UPR. A further analysis of the AT/RT-like tumor expression signature in this context revealed a high expression of UPR related genes. Moreover, we analyzed genes previously identified as upregulated in human AT/RT and also upregulated in MEFs upon deletion of *Smarcb1* and found many of them associated to the UPR. To investigate how SMARCB1 is related to the ER stress response, we knocked down *SMARCB1* in a breast cancer cell line and also compared rhabdoid, SMARCB1 lacking versus non-rhabdoid, SMARCB1 expressing human tumor cell lines. We found that loss of SMARCB1 leads to an increased phosphorylation of eIF2alpha, a central component of the UPR. Phosphorylated eIF2alpha enhances apoptosis in combination with inhibition of the proteasome and we found that the treatment of MCF7-*SNF5* knockdown cells, kidney MRT cells and AT/RT cells with a proteasome inhibitor caused increased apoptosis compared to *SMARCB1* expressing control cells.

## Conclusion

In paper two, we show that both the type and the order of certain genetic events occurring in the same pool of cells direct the development of distinct brain tumor subtypes. Furthermore, we provide evidence for the involvement of the UPR in the pathogenesis of AT/RTs.

We generated three different brain tumor types in this study: glioma, CNS PNET and an AT/RT-like tumor. To validate our model system, we performed a GSEA that revealed a correspondence of the expression profiles of the murine tumors with the

expression signatures of the respective human tumors. Tumor formation was achieved by transplantation of TP53 deficient cells over-expressing *HRAS*, *MYC* or *HRAS+MYC*. These genetic events are observed in the pathology of the respective human tumors, too: *TP53* is considered the most important tumor suppressor and is activated as a cellular response to active oncogenes. It is often inactivated in high-grade gliomas and altered in CNS PNET and AT/RT. *HRAS* and *MYC* are potent oncogenes that trigger diverse molecular pathways and thereby activate the cell cycle. Permanent overactivation of these genes in combination with an inadequate tumor suppressor response pushes a cell toward a state of environmental independent proliferation. Increased *HRAS* signalling is a common feature of gliomas (Guha et al., 1997) and *MYC* is frequently amplified in PNETs and AT/RTs (Behdad et al., 2010). Human AT/RTs are characterized by the loss of the tumor suppressor SMARCB1 that we did not detect in the AT/RT-like murine tumors. However, we observed similarities to human AT/RTs in the gene expression pattern, which indicates that different genetic events can produce a similar tumor phenotype. Although for *Bmi1* and *Ezh2*, two PcG proteins, and *FoxM1*, a cell cycle regulator, elevated levels in human gliomas have been shown (Abdouh et al., 2009; Liu et al., 2006), no contribution to the basic tumor phenotype was observed for these genes. It has been demonstrated that shRNA mediated knockdown of *Bmi1* and *Ezh2* in cancer stem cells and of *FoxM1* in a malignant glioma cell line reduces the tumorigenic potential of the respective cells (Abdouh et al., 2009; Liu et al., 2006; Suva et al., 2009). Furthermore, elevated FOXM1 levels have been reported to promote growth and angiogenesis in gliomas (Dai et al., 2007; Zhang et al., 2008). Together with our results, this data points toward a role in tumor maintenance and progression rather than tumor initiation for *Bmi1*, *Ezh2* and *FoxM1*.

It has been reported previously that the insertion of the *v-HRAS* gene into a small cell lung cancer cell line that over-expressed *MYC* induced a phenotypical transition to large cell undifferentiated lung carcinoma *in vitro* whereas this transition failed upon *v-HRAS* insertion in cells without *MYC* over-expression (Mabry et al., 1988). Here, we showed the transformation of a *MYC* established PNET tumor into an AT/RT-like tumor by *HRAS* over-expression while *HRAS* induced gliomas remained stable upon *MYC* over-expression. The finding indicates that certain tumors that developed upon elevated *MYC* signalling are phenotypically not stable. An explanation for the *MYC*-related tumor plasticity might be its support of an open state of chromatin. In conclusion, we demonstrate that a tumor type is determined by the parameters cell-of-origin, genetic and epigenetic events and the order of genetic events.

In the second part of paper two, we demonstrate that the AT/RT-like tumors we generated in our mouse models resemble human AT/RTs and MRTs with respect to histologic features, overall genetic signatures and the expression of an UPR associated gene set. The UPR comprises three branches that serve primarily to re-establish cell homeostasis after stress conditions like hypoxia and glucose deprivation that lead to

the accumulation of misfolded proteins and a "clogging" of the ER. In our model, we over-expressed *MYC* and *HRAS*, two strong oncogenes that induce a very rapid cell growth and that are known to enhance protein translation and to induce the UPR (Denoyelle et al., 2006). Activation of the UPR may allow the cell to cope with ER stress but may also induce apoptosis if the ER stress is further increased.

We demonstrate a new role of *SMARCB1* -which was so far known as a tumor suppressor and for being involved in chromatin remodelling- in the induction of the UPR: our results suggest that loss of *SMARCB1* leads to an elevated eIF2alpha phosphorylation, which has been shown to protect tumor cells from stress conditions (Bi et al., 2005; Harding et al., 1999; Koumenis et al., 2002; Muaddi et al., 2010; Wiseman et al., 2005). Tumor cells might benefit from the loss of *SMARCB1*, which increases the activity of the catalytic subunit of protein phosphatase-1 (PP1c) that dephosphorylates eIF2alpha. We finally show that the hyperphosphorylation of eIF2alpha renders cells sensitive to further ER stress. We observed increased apoptosis of *SMARCB1* lacking tumor cells that were treated with the proteasome inhibitor Bortezomib, a finding that might represent a therapeutic approach to treat AT/RTs.

## Perspective

*MYC* is a master regulator of gene regulation and activates or represses thousands of target genes. It has key functions in the formation of several highly aggressive and to date incurable brain tumor types. A better understanding of its major molecular functions may lead to the identification of new drug targets and thus provide a basis for a fundamental therapeutic approach.

A gene expression analysis with samples of the generated murine PNETs revealed several genes that were upregulated upon *MYC* over-expression and might contribute to the effect of *MYC*. A subsequent study should focus on the identification of direct *MYC* targets that could be tested for their potential to induce oncogenic transformation in a transduction and transplantation approach similar to the one described in paper two.

AT/RTs represent highly aggressive CNS tumors of infants and children with a very poor prognosis. We demonstrate here that treatment of AT/RT and MRT cells with the proteasome inhibitor Bortezomib induces apoptosis. Future studies should investigate the therapeutic potential of this finding. While in paper two a significant effect of Bortezomib treatment is shown *in vitro*, the next experimental step should consider establishing a stable *in vivo* model system. In future experiments, immunodeficient mice could be transplanted with AT/RT and rhabdoid tumor cells. From a certain stage of tumor development on, the transplanted mice could be treated with Bortezomib or other proteasome inhibitors and monitored for tumor progression.

## Paper 3

Identification of novel BMI1 targets in neural stem/progenitor cells.

### Rationale

BMI1 is a polycomb group protein and acts as a transcriptional repressor by means of chromatin changes. While the expression of *Bmi1* is necessary for the maintenance of the murine hematopoietic and nervous stem cell pools, BMI1 was discovered due to its transformation-supportive role in the generation of B-lymphoid tumors (Haupt et al., 1991; van Lohuizen et al., 1991). It is found over-expressed in several cancer types and is essential for the maintenance of human brain tumors and the oncogenic *in vivo* properties of GBM cells (Abdouh et al., 2009). Few direct BMI1 targets such as the tumor suppressor genes *Ink4a*, *Arf* and *Cdkn1a* have been identified so far and may account for some but not all of the BMI1 effects (Bruggeman et al., 2007; Bruggeman et al., 2005; Fasano et al., 2007; Molofsky et al., 2005; Molofsky et al., 2003).

In paper three, we investigated the effect of *Bmi1* over-expression on neural stem/progenitor cells and tested the oncogenic potential of these cells *in vivo*. Furthermore, we compared the expression profiles of neural stem/progenitor cells expressing elevated *Bmi1* levels and control cells and sought to identify novel direct BMI1 target genes.

### Results

To investigate the effect of *Bmi1* over-expression on neural stem/progenitor cells, we performed a neurosphere (NSP) assay with cells isolated from the postnatal lateral ventricle wall that were transduced with a retroviral vector. We measured higher NSP frequencies and total cell numbers for the *Bmi1* over-expressing cultures compared to empty vector (EV) control cells, thus indicating higher self-renewal and proliferation upon *Bmi1* over-expression. Moreover, NSPs that developed from *Bmi1* over-expressing cells were significantly larger than the controls. To elucidate these effects, we assessed the proliferative and survival capacity of the *Bmi1* over-expressing and EV cells, respectively. We detected both an enhanced survival and an increased proliferation upon *Bmi1* over-expression. However, we did not observe tumor formation when intracranially transplanting *Bmi1* over-expressing cells into mice. These findings indicate that high levels of BMI1 confer a growth advantage but do not render neural stem/progenitor cells tumorigenic. For a better understanding of the molecular changes induced by *Bmi1* over-expression, we compared the gene

expression profiles of *Bmi1* over-expressing versus EV control cells and found 200 genes down- and 100 genes upregulated upon *Bmi1* over-expression. A GSEA performed with the overall gene expression data of *Bmi1* over-expressing and EV cells revealed an enrichment of two embryonic stem cell signatures for the *Bmi1* over-expressing compared to EV control cells, thus indicating the induction of a more immature stem cell phenotype upon *Bmi1* over-expression. In the final part of this study, we applied the data gained from the expression profiling to focus on the search for hitherto unknown direct BMI1 targets. We could identify four genes, namely *Ndn*, *EphA7*, *Rps6ka6* and *Trp53bp2*, which are downregulated upon *Bmi1* over-expression and genomic regions of which are bound by BMI1.

## Conclusion

In paper three, we report a strong growth effect of *Bmi1* over-expression on cultivated neural stem/progenitor cells, namely a higher self-renewal rate, increased proliferation and a reduction of cell death. These observations may be explained by the fact that two major targets of BMI1, the tumor suppressor genes *Ink4a* and *Arf*, are upregulated under cell culture conditions and that high BMI1 levels counteract this "cell culture stress" effect (Sherr et al., 2000). Despite the achievement of such oncogenic features *in vitro*, *Bmi1* over-expressing cells failed to initiate tumor formation when transplanted intracranially into mice, a finding which is in line with previous studies (He et al., 2009; Yadirgi et al., 2011). BMI1 has been described as a cooperator of MYC and RAS (Datta et al., 2007; Haupt et al., 1991; van Lohuizen et al., 1991) and has been associated to the pathogenesis of glioma, medulloblastoma and neuroblastoma (Abdouh et al., 2009; Bruggeman et al., 2007; Bruggeman et al., 2005; Cui et al., 2007; Michael et al., 2008; Molofsky et al., 2005; Molofsky et al., 2003; Wiederschain et al., 2007). In this context, our observation indicates a supportive role of BMI1 for transformation events that are induced by other oncogenes. This hypothesis is corroborated by the GSEAs performed in this study, which revealed an enrichment of genes related to ES cell expression signatures in the *Bmi1* over-expression profile. The BMI1-induced shift of neural stem/progenitor cells to a more immature, ES cell like state might facilitate a neoplastic transformation in cooperation with the activation of other oncogenes.

Based on our gene expression analysis, we could identify several new target genes of BMI1, of which four were shown to be directly bound by BMI1. *Ndn* encodes a potential tumor suppressor (Chapman et al., 2009) that participates in the regulation of *BMI1* via E2F (Chapman et al., 2008; Kuwako et al., 2004) and has been found upregulated in a *BMI1* knockdown study (Douglas et al., 2008). The product of *EphA7* is a signalling receptor involved in regulation of apoptosis of neural progenitors (Depaepe et al., 2005); *Rps6ka6* encodes a tumor suppressor associated with stress-dependent and replicative senescence (Lopez-Vicente et al., 2009). The

product of *Trp53bp2* is an enhancer of pro-apoptotic functions of TRP53 (Vives et al., 2006) and has also been reported to maintain cell polarity (Sottocornola et al., 2010).

## Perspective

Future research based on the present study might focus on the question how strong the contribution of the identified direct BMI1 target genes to the described *Bmi1* over-expression effect on neural stem/progenitor cells is.

To address this question, the expression of the identified target genes could be repressed in neural stem/progenitor cells. The manipulated cells could be used for neurosphere, proliferation and apoptosis assays to determine their growth properties and to compare these results to *Bmi1* over-expressing neural stem/progenitor cells. Repression of gene transcription can be achieved either by shRNA-mediated knockdown or complete deletion of the gene in mice. While both options are possible, the first method would presumably better resemble the BMI1-mediated transcription repression effect, as it does not completely inhibit the transcription of a gene but reduces it. In a similar way, knocking down a gene with shRNA vectors will, depending on the efficacy of the respective applied construct, yield a more or less strong reduction of transcription.

# CONCLUDING REMARKS

In paper one, we investigated how the pharmacological activation and inhibition of the glucocorticoid system affects lifespan and symptoms in a mouse model for RTT. We performed a long-term drug treatment study with the GR activator corticosterone and the GR inhibitor RU486 under which we measured the lifespan and onset of RTT-like symptoms of male *Mecp2*-null and female *Mecp2* heterozygous mice in comparison to untreated mutant and to treated and untreated wild-type animals. We could demonstrate that activation of the glucocorticoid hormone system reduces the lifespan of *Mecp2*<sup>ly</sup> mice and the symptom-free lifetime of *Mecp2*<sup>+/-</sup> mice and that treatment with the GR inhibitor RU486 has an opposite effect as it prolongs the lifetime until symptom onset for *Mecp2*<sup>+/-</sup> mice and improves motor functions of *Mecp2*-null male mice. Our findings provide evidence for the contribution of the glucocorticoid hormone system to RTT motor symptoms and suggest this system as a potential therapeutic target for RTT.

In paper two and three, we focused on the molecular events that lead to the development of primary malignant brain tumors.

In paper two, we performed a series of transplantation experiments with genetically perturbed cells. We could show that the individual over-expression of potent oncogenes in neural stem/progenitor cells of the same cell pool leads to distinct tumor types. Furthermore, we demonstrated that it is possible to convert one tumor type into another one and that this is determined by the order of genetic events. In a second part of this study we could show a hitherto unknown aspect of AT/RT and rhabdoid tumor biology, an activation of the UPR. We provide experimental evidence that AT/RT and rhabdoid tumor cells with reduced or absent SMARCB1 levels are sensitive toward a further increase in ER stress. The latter finding might be therapeutically explored.

In paper three, we studied the PcG protein BMI1 and its effect on neural stem/progenitor cells and tumor formation. We observed a strong promotion of self-renewal, expansion and survival in adult neural stem/progenitor cells upon over-expression of *Bmi1* *in vitro* but found it incapable of transforming cells as no tumors developed in intracranial transplantation experiments with *Bmi1* over-expressing wild-type cells or *Trp53*<sup>-/-</sup> cells (paper two). Based on our results and the findings of other groups, we assume BMI1 to promote stem cell properties and to act as a facilitator of transforming events induced by other oncogenes. Furthermore, we could identify four novel direct BMI1 target genes whose molecular function may contribute to the known BMI1 effects, thus expanding the BMI1 network.



Taken together, the findings presented in this thesis emphasize the key role of master regulators in the pathology of brain diseases and highlight the importance of elucidating their molecular effects for the development of causal therapies.

# KEY METHODS OF THIS THESIS

## Neurosphere assay

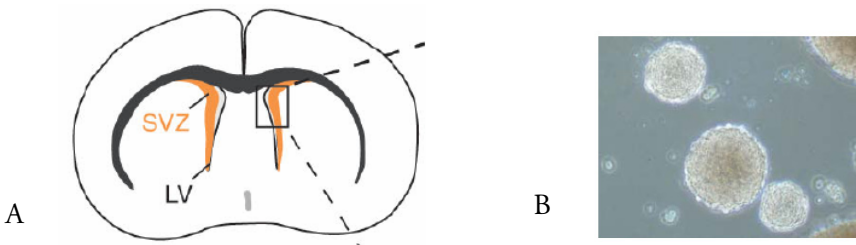
NSPs represent three-dimensional floating spheroid cell clusters that form *in vitro* from neural stem and progenitor cells isolated from the developing or adult mammalian CNS (Figure 7) (Jensen et al., 2006; Singec et al., 2006). The NSP culture system was introduced by Reynolds and Weiss who first cultured cells with stem cell properties as free-floating cultures by dissecting striatal tissue that included the SVZ (Pastrana et al., 2011; Reynolds et al., 1992). In the presence of EGF, a small cell population formed spheres of proliferating cells with the capacity to form secondary spheres upon passaging and to differentiate into neurons and glial cells, thus providing the first *in vitro* evidence that multipotent stem cells are present in the adult mammalian brain.

Continuous neurogenesis in the adult mammalian brain mainly occurs in two regions, namely the SVZ, which is a thin layer of dividing cells adjacent to the lateral ventricles, and the subgranular zone (SGZ) in the hippocampal formation. These areas harbor stem cells that retain the capacity to self-renew and differentiate into the three primary cell types of the brain: neurons, astrocytes and oligodendrocytes. While SGZ cells can form NSPs, dissociation and replating does not lead to secondary spheres or only generates spheres until the third passage (Pfenninger et al., 2007). SVZ cells can be passaged up to 10 times (Bull et al., 2005; Pfenninger et al., 2007; Reynolds et al., 1996; Seaberg et al., 2002). Based on these differences and the fact that SGZ cells do not differentiate into the three neural lineages, adult neurogenic SGZ cells are called progenitors, and long-term passagable, NSP-forming adult SVZ cells with multilineage potential are termed stem cells (Morshead et al., 2004; Pfenninger et al., 2007). Moreover, it has been shown that NSPs cannot only be generated from brain areas of continuous neurogenesis but from the entire ventricular axis of the adult CNS, including the spinal cord (Vescovi et al., 1993; Weiss et al., 1996). While both EGF and bFGF are required to grow NSPs from non-neurogenic regions, EGF NSPs can only be cultured from the SVZ (Pastrana et al., 2011).

It has become clear that EGFR-expressing cells that are in a proliferative state, namely activated GFAP-positive (type B) stem cells and transit-amplifying (type C) cells, function as neurosphere-forming cells *in vitro*, with the capacity to be serially passaged and the feature of multipotency (Pastrana et al., 2011). In this context, it is important to note that putative quiescent stem cells do not give rise to NSPs and are

not detected when the self-renewal capacity of a cell population is determined with the NSP assay. Therefore, the NSP assay cannot be used alone to define the *in vivo* stem cells. However, it represents a simple tool to analyze the multipotency, self-renewal capacity and proliferation of neural stem/progenitor cell populations *in vitro*.

A central tenet for the interpretation of an NSP assay is clonality, i.e. the derivation of each NSP from one single cell, which is determined by the applied cell density. This issue has been discussed controversially and a wide range of ratios of cells per volume of medium is regarded to be consistent with clonal conditions (Pastrana et al., 2011). However, mixing experiments suggest a reliable clonality only upon a density of 1 cell/ $\mu\text{l}$  when using passaged NSPs (Coles-Takabe et al., 2008). Still, NSP culture artifacts like aggregation of spheres and cultured cells that might lead to an incorrect readout have to be taken into account. Therefore, true clonality can only be guaranteed by plating single cells per well. However, such an approach comes with the restriction that plating of single cells decreases the sphere-forming efficacy significantly due to paracrine signals released by cells into the medium and is not always applicable (Pastrana et al., 2011).



### Figure 7. Culturing of NSPs.

A, coronal section of the murine brain with the lateral ventricles. The site used for tissue isolation in order to generate NSPs is indicated (figure taken from Doetsch, 2003). B, appearance of NSPs *in vitro*.

## Retroviral over-expression system

Retroviral gene transfer has been developed as an efficient method to target dividing cells and to induce stable, inheritable expression of one or more desired genes by integration of the respective coding sequence, controlled by a suitable promoter, into the genome of the host cell. In this thesis, retroviral constructs coding for the cDNA sequence of the gene of interest and cis-acting viral elements including the CMV

promoter, a polyadenylation signal, the viral packaging signal  $\Psi$  and viral long terminal repeats (LTR; see (Coffin et al., 1997) for details) were used. Between the LTRs, i.e. the sequence part to be integrated into the host cell genome, these constructs featured the coding sequences for a reporter gene and the gene of interest, separated by an internal ribosomal entry site (IRES). Due to this bi-cistronic composition of the constructs, the co-transcription of the reporter gene with the gene of interest was ensured. A packaging cell line was transiently transfected with the retroviral constructs to produce viral particles. As the viral structure genes *gag*, *pol* and *env* were not included in the vector construct sequence but integrated in the genome of the packaging cell line, transduction competent/replication incompetent retroviruses were produced. The retroviral host range is determined by *env*, which encodes the envelope protein. We generated ecotropic viral particles that only infect murine cells. The viral particles were harvested from the culture supernatant of the transfected packaging cells and mixed with freshly passaged NSP cells for transduction. After one week, the formed NSPs were digested and positive transductants were FACS-sorted based on the expression of the respective fluorescent reporter gene.

## Fluorescence activated cell sorting (FACS)

FACS is a technique that is used to measure and analyze physical properties of single cells as they pass through a beam of laser light. The measured properties include the cells size, granularity and fluorescence emission and are determined with an optical-to-electronic system (Gautho, 2003).

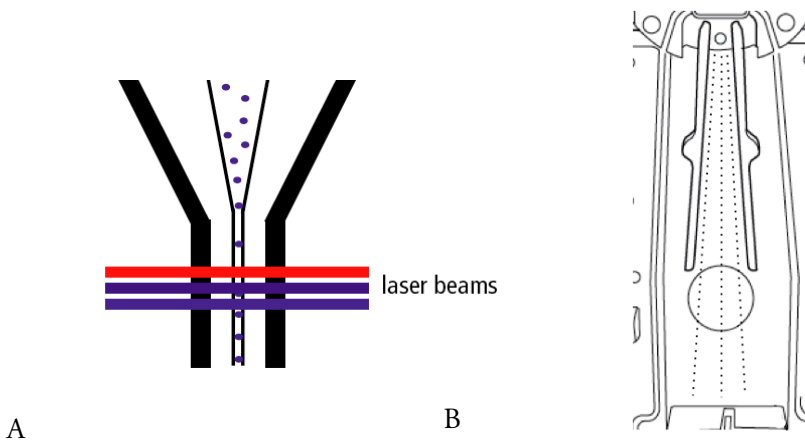
Basically, a FACS system that is equipped with a sorting device consists of three main systems:

- The fluidic system that transports cells in a fluid stream to the laser interrogation point and the drop drive that generates distinct droplets containing single cells that can be deflected (Figure 8).
- The optics system that consists of lasers that excite the fluorochromes and optical fibers that transport the emission signal to appropriate detectors.
- The electronic system that converts the light signals into electronic signals that are processed with a computer and result in a sort decision.

When a cell intercepts a laser beam, light is scattered, depending on the size and internal complexity of the cell. While forward-scattered light (FSC) is a measure for the size of a cell, side-scattered light (SSC) is proportional to its granularity. Correlated FSC and SCC are used to determine homogenous, defined populations among a heterogeneous pool of cells.

The great potential of cell sorting systems is its capacity to characterize subgroups of cells according to their respective phenotype and to isolate these cells as a highly enriched population. The marking of cells is based on fluorescent dyes and can be achieved by a) labeling prior to FACS analysis via incubation with surface protein specific, fluorochrome coupled antibodies or dyes or b) expression of a fluorescing protein like eGFP or dsRed. A FACS system typically contains several lasers that emit light at distinct wavelengths (e.g. 488 nm and 633 nm) and excite the fluorochromes when a cell intercepts the laser light, thus generating an emission signal that is detected and analyzed.

Based on the respective emission signals, a sort decision is computed. Cell-containing droplets emitting the desired fluorescence signals are electrically charged and deflected in an electric field while non-target cell droplets do not get charged. Thereby, defined cell populations can be separated from each other and collected for further processing.



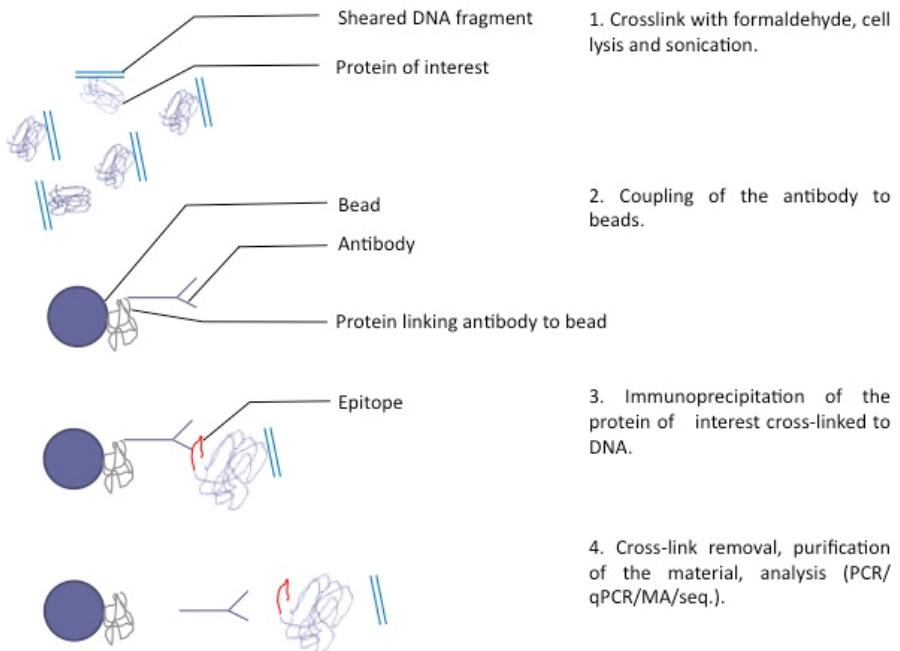
**Figure 8. Principles of FACS.**

A, hydrodynamic focusing of the sample core through the flow cell in which the cells pass the laser beams. B, deflection plates in a sorting block of a BD Aria cell sorting system. The deflection plates generate an electric field in which cell-containing droplets are directed toward collection tubes or waste, depending on their electrical charge (figures taken from Gautho, 2003).

## Chromatin immunoprecipitation (ChIP)

ChIP is a powerful method and widely applied technique to study interactions of DNA with proteins like histones or transcription factors (Aparicio et al., 2005; Dahl

et al., 2008). Briefly, cells are treated with formaldehyde to generate a cross-link between DNA and protein (Figure 9). A whole-cell extract is prepared and the cross-linked DNA is sheared by sonication, resulting in DNA fragments of about 500 bp. The material is immunoprecipitated using a suitable antibody directed against the protein of interest and coupled to beads that can be used for separation by centrifugation or magnetism. Thus, DNA sequences selectively binding to a certain protein are enriched. After enzymatic removal of the cross-link, the purified DNA can be used as template for PCR reactions to amplify anticipated genomic binding regions. Instead of classic PCR, it is possible to apply quantitative PCR (qPCR) to measure the level of protein-DNA association or to combine the ChIP technique with a microarray (MA) analysis (ChIP-on-Chip) to identify the location of proteins on a genome-wide basis. ChIP-sequencing (ChIP-seq.) represents another ChIP-based technique that combines immunoprecipitation with simultaneous DNA sequencing and, in contrast to ChIP-on-Chip, is not dependent on large sets of probes to allow a high-resolution scan.



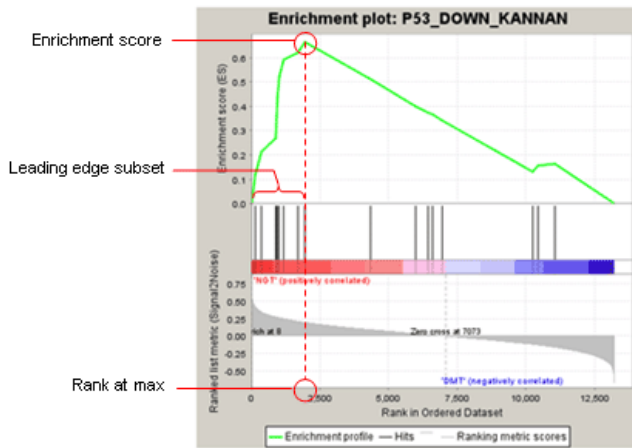
**Figure 9. Main steps of ChIP.**

## Gene set enrichment analysis

The simultaneous analysis of genomewide gene expression levels of thousands of genes with DNA microarrays has become a standard technique in molecular cell biology. While it is now possible to acquire detailed gene expression data in relatively short time, a challenge lies in the interpretation of these data and the recognition of signatures and patterns.

GSEA is a method that evaluates gene expression data at the level of gene sets (Subramanian et al., 2005), which are defined according to published information. Basically, if the gene expression data for two related samples (e.g. cells over-expressing gene  $x$  vs. non-over-expressing control cells) is ordered in a ranked list  $L$  according to the respective expression, GSEA tries to determine if members of gene set  $S$  tend to occur toward the top or bottom of  $L$ , i.e. the group of most differentially expressed genes (Figure 10). If this is the case,  $S$  is correlated to the phenotype of the sample of interest. GSEA consists of three key elements: I. An enrichment score ( $ES$ ) is calculated that reflects to which extent  $S$  is represented at the top or bottom of  $L$ : walking down  $L$ , it is increased when a gene in  $S$  is encountered and decreased when genes are not in  $S$ . The magnitude of the  $ES$  describes the correlation between the phenotype of the sample and  $S$ . II. The significance level of the  $ES$  is calculated with an empirical phenotype-based permutation test procedure. The  $ES$  is recomputed with permuted phenotype labels, thus generating an  $ES$  null distribution to which the  $P$  value of the observed  $ES$  is then calculated. III. To enable the comparison of several gene sets and multiple hypothesis testing, the  $ES$  of each gene set is normalized ( $NES$ ) and the proportion of false positives is determined by calculation of the false discovery rate (FDR) that corresponds to each  $NES$  (Subramanian et al., 2005).

Compared with single-gene methods, GSEA provides several advantages: it makes the handling of huge data sets easier and helps with the identification of signatures and patterns, thus revealing functional information. It also makes it possible to identify modest expression changes in groups of functionally related genes that otherwise might be lost. Finally, the leading-edge analysis helps to define gene subsets that might be relevant for the phenotypical description of a specimen.



**Figure 10. Example for a GSEA enrichment plot with indicated ES, leading edge subset and maximum rank.**

(figure taken from Subramanian et al., 2005)





# POPULAR SCIENCE SUMMARY

The human brain is a complex organ that can be considered as a network of highly specialized and interacting cells. Correct spatial and temporal proliferation, development and maintenance of the individual cells of this network are prerequisites for proper brain functioning and depend on a stringent cellular gene expression control. This regulation system can be imagined as a hierarchy: so called master regulators on top control the expression of a large number of effector genes. If these key regulators are lost or permanently activated, expression regulation changes significantly, cells respond inappropriately to environmental signals, and the brain may enter a diseased state.

This thesis deals with two types of severe brain diseases, Rett syndrome (RTT) and primary malignant brain tumors, and the effects of single master regulators in these disorders: MeCP2 in case of RTT and MYC, RAS and BMI1 in case of brain tumors. We focused on the molecular alterations that occur upon changes in the activity of such regulators to contribute to an improved understanding of the pathomechanism of these diseases.

RTT is a disease that mainly occurs in girls and affects their neurological development, leading to physical and mental impairment. Most cases are caused by the loss-of-function of MeCP2, a protein that acts as a regulator of gene expression in the brain. Based on the previous finding that MeCP2 controls two genes that are co-regulated by the glucocorticoid hormone system, we investigated in the first study of this thesis how a pharmacological activation and inhibition of this system influences symptoms in a mouse model that mimics human RTT. We monitored drug-treated mice regularly and observed that the activation of the glucocorticoid hormone system has a negative impact on lifespan and motor symptoms of the mice while an inhibition of this hormone system increases the symptom-free lifetime of these animals and their performance in motor tests. Our results provide further evidence for the involvement of this hormone system in RTT and suggest the inhibition of this system as a potential therapeutic avenue for RTT.

In studies two and three, we investigated molecular mechanisms that confer a cell the potential to generate a tumor, i.e. the potential for an oncogenic transformation. Since there is growing evidence that brain tumors originate from cells with stem/progenitor cell features, we used neural stem/progenitor cells as a model system for our experiments.

Despite intensive research during the last decades, treatment options to counter-act aggressive malignant brain tumors remain poor. To gain a better understanding of the

background of brain tumor development, we genetically perturbed neural stem/progenitor cells and performed serial brain transplantation experiments with mice in study two. Transplanted animals developed highly aggressive brain malignancies that resemble human tumors mainly affecting young patients: giant cell gliomas, CNS primitive neuroectodermal tumors (PNETs) and atypical teratoid/rhabdoid-like (AT/RT-like) tumors. Since all tumors were generated from the same pool of cells, the respective tumor type was dependent on the specific genetic perturbations and the order of these genetic events as we could convert PNET cells, but not glioma cells into AT/RT-like tumor forming cells by applying the same genetic alterations in a different order. Performing a detailed analysis of the expression data of the generated AT/RT-like tumors, we discovered the activation of the unfolded protein response (UPR), a protective mechanism that helps cells to deal with high amounts of unfolded proteins in the endoplasmic reticulum (ER). An activated UPR was also observed in cell lines of human AT/RT and mouse fibroblasts that lacked expression of the tumor suppressor gene *Smadcb1*. The further elevation of ER stress in these cells by inhibition of the cellular protein degradation machinery led to increased cell death. The latter finding might represent a therapeutical approach for the treatment of AT/RTs or malignant rhabdoid tumors occurring in organs other than the brain and should be investigated in future studies.

In study three, we focused on BMI1, a protein that is necessary for a proper development of the blood- and the central nervous system and which is also associated with the development of several types of cancer. Upon increased levels of BMI1, neural stem/progenitor cells showed significantly higher self-renewal, expansion and survival. However, both wild-type and neural stem/progenitor cells lacking the tumor suppressor gene *Trp53* (paper two), despite over-expressing *Bmi1*, failed to generate tumors when we transplanted them into mouse brains. Together with findings of other groups, our data suggest that BMI1 facilitates cell transformation by other oncogenes via inhibition of an appropriate cellular tumor preventing response and the creation of a transformation supporting cell state. Moreover, we were able to identify hitherto unknown genes that are directly regulated by BMI1 and whose repression might explain the *Bmi1* over-expression effect that we observed. Thus, our results contribute to an expansion of the molecular BMI1 network.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Den mänskliga hjärnan är ett komplext organ som kan anses som ett nätverk av högt specialiserade och interagerande celler. Den korrekta spatiala och temporala proliferationen, utvecklingen och upprätthållandet av nätverkets individuella celler är förutsättningar för en fungerande hjärna och beror på en stringent cellulär genuttrycks kontroll. Detta regulationssystem kan föreställas som en hierarki: så kallade masterregulatorer på spetsen kontrollerar uttryckningen av ett stort antal effektorgener. Om dessa masterregulatorer saknas eller aktiveras permanent kommer genuttrycks kontrollen att förändras signifikant, celler slutar svara lämpligt på miljösignaler och sjukdomar kan utvecklas.

Denna avhandling handlar om två svåra hjärnsjukdomar, Rett syndrome (RTT) och primära maligna hjärntumörer, och effekterna av enstaka masterregulatorer i dessa störningar: MeCP2 i fallet av RTT och MYC, RAS och BMI1 i fallet av hjärntumörer. Vi koncentrerade oss på de molekylära förändringarna som förekommer i celler vars genuttryckning är felreglerad för att utvidga kunskapen om patogenitetsmekanismen av dessa sjukdomar.

RTT är en sjukdom som drabbar framförallt flickor och påverkar deras neurologiska utveckling. RTT ackompanjeras av en fysisk och mental utvecklingsstörning och orsakas huvudsakligen av en funktionsförlust av MeCP2, ett protein som agerar som en regulator av hjärnans genuttryckning. Grundande på den tidigare upptäckten av att MeCP2 kontrollerar två gener som även regleras av glukokortikoidhormonsystemet, så utredde vi i avhandlingens första del hur en farmakologisk överaktivering och hämning av glukokortikoidsystemet påverkar symptomen i en musmodell för RTT. Vi observerade att en aktivering av glukokortikoidsystemet har en negativ effekt på mössens livslängd och motoriska symptom medan en hämning av detta hormonsystem förhöjde den symptomfria livstiden och förbättrade prestationen i motoriska test. Våra resultat bekräftar en inblandning av detta system i RTT och tyder på en hämning av systemet som en möjlig terapeutisk väg för RTT.

Under studierna två och tre undersökte vi molekylära mekanismer som leder till utveckling av cancer celler och hjärntumörer. Eftersom det finns växande bevis att hjärntumörer härstammar från celler med stamcellegenskaper använde vi neurala stam/progenitor celler som ett modellsystem under våra experiment.

Trots en intensiv forskning under dem senaste decennierna förblir de terapeutiska möjligheterna för aggressiva maligna hjärntumörer ytterst begränsade. För att bättre förstå bakgrunden av hjärntumörutvecklingen förändrade vi under studie två neurala stam/progenitor celler genetiskt och genomförde seriella hjärntransplantationsexperiment med möss. Transplanterade djur utvecklade ytterst aggressiva tumörer som kan jämföras med tumörer hos människor vilka huvudsakligen drabbar unga patienter: jättecellgliomas, CNS primitiva neuroektodermala tumörer (PNETs) och atypiska teratoida/rhabdoida- (AT/RT-) liknande tumörer. Eftersom alla tumörer genererades av samma cell pool avgjordes de respektive tumörtyperna av de genetiska förändringarna och av ordningen av dessa modifikationer: vi kunde omvandla PNET celler men inte glioma celler till AT/RT-liknande tumörigena celler vid samma modifikationer i olik ordning. Genom en noggrann undersökning av genuttrycksprofilen av de genererade AT/RT-liknande tumörerna upptäckte vi en aktivering av den "unfolded protein response" (UPR), en skyddsmekanism som hjälper celler att hantera höga proteinmängder i endoplasmatiska retikulum (ER). En aktiverad UPR observerades också i cellinjer av human AT/RT och mus fibroblaster som saknade uttryckningen av tumörsuppressorgen *Smarch1*. När ER stress ökades i dessa celler genom en hämning av det cellulära proteinnedbrytningsmaskineriet upptäckte vi en förhöjd celldöd. Denna upptäckt skulle kunna föreställa en terapeutisk väg för att behandla AT/RTs och maligna rhabdoida tumörer som förekommer i andra organer än hjärnan och borde undersökas under kommande studier.

Under studie tre fokuserade vi på BMI1, ett protein som är nödvändigt för en korrekt utveckling av blod- och det centrala nervsystemet och vilket är samtidigt inblandat i utvecklingen av flera typer av cancer. Vid förhöjda mängder av BMI1 visade neurala stam/progenitor celler en signifikant förhöjd självförnyelse, utökning och överlevnad. Både vildtypiska och neurala stam/progenitor celler som saknade tumörsuppressorgen *Trp53* (studie två) förmådde dock inte att generera tumörer vid transplantation i mushjärnor trots en överuttryckning av *Bmi1*. Tillsammans med andra studier visar våra resultat att BMI1 underlättar en transformation genom att förhindra ett lämpligt, anti-onkogen cellulär svar och generera ett transformationsstödjande celltillstånd. Dessutom har vi kunnat identifiera hittills okända gener som regleras direkt av BMI1 och vars funktionella hämning skulle förklara *Bmi1* överuttryckningseffekten som vi observerade. Våra resultat bidrar därmed till en utökning av det molekylära BMI1 nätverket.

# POPULÄRWISSENSCHAFTLICHE ZUSAMMENFASSUNG

Das menschlich Gehirn ist ein komplexes Organ, das als Netzwerk hoch spezialisierter und interagierender Zellen begriffen werden kann. Die korrekte räumliche und zeitliche Vermehrung, Entwicklung und Erhaltung der individuellen Zellen dieses Netzwerkes sind Grundbedingungen für dessen einwandfreies Funktionieren und abhängig von einer präzisen zellulären Genexpressionskontrolle, die man sich als hierarchisch aufgebaute Pyramide vorstellen kann: sogenannte Masterregulatoren an der Spitze kontrollieren die Expression zahlreicher Effektorgene. Fehlen diese Schlüsselregulatoren oder sind sie permanent aktiviert, führt das zur Veränderung der Genexpressionskontrolle, inadäquaten Antworten der Zellen auf Umweltsignale und möglichen Entwicklung von Krankheiten.

Gegenstand dieser Arbeit sind zwei schwere Erkrankungen des Gehirns, Rett Syndrom (RTT) und primäre maligne Hirntumore, und die Effekte einzelner Masterregulatoren bei diesen Störungen: MeCP2 im Fall von RTT und MYC, RAS und BMI1 im Fall von Hirntumoren. Wir konzentrierten uns auf die molekularen Veränderungen in Zellen mit veränderter Genexpression um ein besseres Verständnis von den Krankheitsmechanismen dieser Erkrankungen zu erhalten.

RTT ist eine Erkrankung, die vor allem bei Mädchen auftritt. Die neurologische Entwicklung betroffener Patienten ist beeinträchtigt und die Erkrankung geht mit physischen und mentalen Entwicklungsstörungen einher. RTT wird in den meisten Fällen durch den Funktionsverlust von MeCP2, einem Protein, das als Genexpressionsregulator im Gehirn fungiert, verursacht. Basierend auf einer früheren Studie, nach der MeCP2 zwei Gene kontrolliert die vom Glukokortikoidhormonsystem reguliert werden, untersuchten wir im ersten Teil dieser Arbeit, wie eine pharmakologische Aktivierung bzw. Hemmung des Glukokortikoidsystems die Symptome eines Mausmodells für RTT beeinflusst. Wir beobachteten, dass eine Aktivierung des Glukokortikoidhormonsystems einen negativen Einfluss auf das Alter und die motorischen Symptome der Mäuse hatte, während eine Hemmung dieses Hormonsystems eine verlängerte symptomfreie Lebenszeit der Tiere und verbesserte Ergebnisse in motorischen Tests bewirkte. Unsere Ergebnisse geben weitere Anhaltspunkte für eine Beteiligung dieses Hormonsystems bei RTT und regen die Hemmung dieses Systems als einen potentiellen therapeutischen Weg an.

In den Studien zwei und drei untersuchten wir molekulare Mechanismen die zur Entwicklung von Krebszellen und zur Entstehung von Hirntumoren führen. Da sich die Anhaltspunkte für einen Ursprung von Hirntumoren aus Zellen mit Stammzeleigenschaften verdichten, haben wir für unsere Experimente neurale Stamm-/Vorläuferzellen als Modellsystem benutzt.

Trotz intensiver Forschung während der letzten Jahrzehnte sind die Behandlungsmöglichkeiten aggressiver maligner Hirntumoren dürftig. Um ein besseres Verständnis des Hintergrunds der Hirntumorentwicklung zu erlangen, haben wir in Studie zwei neurale Stamm-/Vorläuferzellen genetisch modifiziert und serielle Hirntransplantationsexperimente mit Mäusen durchgeführt. Die transplantierten Tiere entwickelten hoch aggressive Tumore, die vergleichbar sind mit Entitäten beim Menschen, welche vor allem bei jungen Patienten auftreten: Riesenzellgliome, ZNS primitive neuroektodermale Tumore (PNETs) und atypische teratoid/rhabdoid (AT/RT)-ähnliche Tumore. Alle Tumore wurden aus demselben Zellpool generiert und der jeweilige Tumortyp erwies sich als abhängig von den genetischen Modifikationen und der Reihenfolge dieser Modifikationen -wir konnten PNET-Zellen, nicht aber Glioma-Zellen in AT/RT-ähnliche, tumorigene Zellen umwandeln wenn wir dieselben genetischen Veränderungen in unterschiedlicher Reihenfolge durchführten. Anhand einer detaillierten Genexpressionsanalyse der erzeugten AT/RT-ähnlichen Tumore konnten wir die Aktivierung der "unfolded protein response" (UPR), eines protektiven Mechanismus, der Zellen hilft mit grossen Mengen ungefalteter Proteine im Endoplasmatischen Retikulum (ER) umzugehen, feststellen. Eine aktivierte UPR wurde ausserdem in Zelllinien von humanen AT/RT-Tumoren sowie Maus-Fibroblasten, die *Smcarb1* nicht exprimierten, beobachtet. Wenn der ER-Stress in diesen Zellen durch Hemmung der zellulären Proteindegradationsmaschinerie weiter erhöht wurde, führte dies zu vermehrtem Zelltod. Dieses Ergebnis könnte eine Möglichkeit zur Entwicklung einer künftigen Therapie für AT/RT- sowie nicht im Gehirn vorkommender maligner rhabdoider Tumore darstellen und sollte in künftigen Studien untersucht werden.

In Studie drei haben wir uns auf BMI1 konzentriert, ein Protein das notwendig ist für die korrekte Entwicklung des Blut- und des Zentralnervensystems und gleichzeitig mit der Entwicklung verschiedener Krebsarten in Verbindung gebracht wird. Bei Erhöhung der zellulären BMI1-Konzentration zeigten neurale Stamm-/Vorläuferzellen eine signifikant höhere Selbsterneuerung, Expansion und Überleben. Weder die intrakraniale Transplantation von wildtypischen noch von neuronalen Stamm-/Vorläuferzellen ohne Expression des Tumorsuppressorgens *Trp53* (Studie zwei) in Mäuse führte jedoch zur Entwicklung von Tumoren. Zusammen mit den Ergebnissen anderer Gruppen deuten unsere Daten darauf hin dass BMI1 die Transformation von Zellen durch andere Onkogene erleichtert indem es eine angemessene zelluläre Antwort zur Verhinderung einer Tumorbildung unterdrückt und einen Transformations-unterstützenden Zellzustand schafft. Weiterhin konnten

wir zum gegenwärtigen Zeitpunkt unbekannte Gene, die direkt von BMI1 reguliert werden und deren Unterdrückung den von uns beobachteten *Bmi1* Überexpressionseffekt erklären könnte, identifizieren. Unsere Ergebnisse tragen so zur Erweiterung des molekularen BMI1 Netzwerkes bei.





# ACKNOWLEDGMENTS

All things must pass...

... and even my PhD time came to an end. It was an interesting period of my life and I learned much about how science -but also how life in general- works.

First and foremost I would like to thank my supervisor Ulrike Nuber for providing me with interesting scientific projects, giving me the chance to spend my PhD time in her lab and guiding me through those five years. Ulrike always found the right way to drive the project into the right direction when things were running well but also kept the spirit up in rougher times and encouraged me to continue, coming up with new ideas and suggestions. I found the door to her office always open for troubleshooting and critical, straightforward problem discussion. Even more important, despite the many usual ups and downs of a PhD time, I always had the feeling that this project would come to a positive ending.

I want to thank Olle Lindvall, my co-supervisor, and Isabella Artner, for most appreciated council during thesis advisory committee meetings and more unofficial discussions on the BMC corridors.

Furthermore I would like to express my deep gratitude to Falk Hertwig, both wise teacher and lab buddy during most of my time in the Nuber lab. With never ending patience, he taught me many aspects of scientific work and always knew a way out if experiments were stuck. Skål to the uncounted evenings in front of the Diva cell sorter, rocking the BMC and chatting about the problems of science and the rest of the world.

The Nuber group has always been and still is an interesting mixture of characters and nationalities, and I had the opportunity to work with wonderful people during my PhD time. I always enjoyed the friendly atmosphere in the Nuber group and the possibility to both discuss scientific problems with my lab members and also broaden my horizon about many countries and foreign cultures. Especially, I want to thank Denise Kottwitz who introduced me to the Rett syndrome project and to the work with lab mice. Thanks to Cosima Pfenninger who explained numerous practical and theoretical lab things to me and always was (and still is) my idol of efficiency and discipline in the lab. Thank you to Marcus Winkler for supporting me in final experiments on the BMI1 project during my paternal leave. Also, thank you to Isabelle Leefa for continuing with the Rett syndrome project- and good luck for your future research on this enigmatic disease. Big thank you for a great time to all the past and present Nuber lab members that are not mentioned here.

I want to say thank you to the FACS facility, Teona (Teia) Roschupkina and Zhi Ma, for providing me with theoretical and practical advice on cell sorting and for always, even at strange evening times, answering my numerous questions and coming up with helpful tricks to convince the stubborn machine. Thank you to Eva Gynnstam, Lena Persson Feld and the personal of the konventionella and barriär mouse facilities for always figuring out solutions for my nonstandard wishes and for their reliable, important work.

Many nice people helped me with the experiments of this thesis by providing consumables, introducing me to new techniques and letting me use their technical equipment. Though there is not enough space here to mention all of them, their help is not forgotten and I want to express my gratitude to them.

Finally, a word on the lab mice: in Sweden, every year more than 500.000 mice are used for scientific experiments according to the jordbruksverket, and more than thousand mice have been used for the experiments of this thesis. Although the mouse as a scientific model organism does not optimally reflect the complexity of the human body, it represents, at least at the moment, the only applicable way to learn more about the mechanisms of human life and the pathogenesis of severe human diseases. I am grateful for every gained insight and convinced that the new findings based on the work with lab mice will help to improve human health.

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