Development of brain tumors from neural stem/progenitor cells

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Falk Hertwig

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1. Gutachter: Prof. Dr. Ulrike A. Nuber

2. Gutachter: Prof. Dr. Constance Scharff

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SUMMARY

Brain tumors are neoplasms of the central nervous system (CNS) and are classified according to their histopathology. Current therapies rely on this classification, but the exact factors specifying these tumor types are not known. Identifying the cellular origin and molecular basis of brain tumor development will contribute to the advancement of new targeted therapies.

This thesis investigated the connection between normal neural stem/progenitor cells and brain tumors with respect to the cellular origin of brain tumors and molecular events contributing to their development.

The first part of this work focused on surface markers of neural stem/progenitor cells in the lateral ventricle wall (LVW) and spinal cord (SC), which allowed the prospective isolation and investigation of such cells. CD133 was discussed as a "neural stem cell marker" and a phenotypic hint toward a possible lineage relationship between neural stem cells and CD133-positive brain tumor stem cells. A thorough investigation of neural stem cell types carrying this surface marker was however missing at the beginning of this thesis. In this work, CD133 was identified on two cell types in the postnatal LVW region: post-mitotic ependymal cells – the vast majority of CD133-positive LVW cells – and a subpopulation of ventricle-contacting astrocytic stem cells. In addition, CD133-positive ependymal cells in the adult SC were investigated. In contrast to ependymal cells in the LVW, SC ependymal cells showed *in vitro* self-renewal and multipotency. Comparative gene expression analysis of both cell types revealed that SC ependymal cells express certain genes which likely contribute to their stem cell properties. Furthermore, several genes were found upregulated in SC ependymal cells which have previously been reported as signature genes in ependymomas – i.e. tumors of the SC, which may originate from SC ependymal cells. The observed molecular and functional similarities between self-renewing stem cell types and tumor cells indicate a possible derivation of brain tumors from a normal stem/progenitor cell type.

In the second part of this work, two main questions were addressed: 1) Which oncogenic factors are sufficient to induce brain tumor development from normal neural stem/progenitor cells of the LVW, and 2) Do genetic events direct brain tumor phenotypes? The combination and order of *HRAS* and *MYC* over-expression in *Trp53*-deficient neural stem/progenitor cells was found to instruct the development of gliomas, CNS PNETs or atypical teratoid/rhabdoid (AT/RT)-like tumors. AT/RT-like tumors histologically resembled human AT/RTs and the gene expression profile of both of these tumors indicated an activation of the unfolded protein response (UPR). This cellular pathway is induced upon stress conditions like hypoxia or nutrient-deprivation and has an essential and supportive role for rapidly growing tumors like AT/RTs. AT/RTs are characterized by the loss of function of the tumor suppressor SMARCB1 and investigations in this thesis demonstrate that this loss leads to an increased sensitivity toward eIF2alpha phosphorylation, which is a central UPR component. Based on these findings, an interference with the UPR is suggested as a novel strategy for the treatment of SMARCB1-deficient tumors. In contrast to *MYC* and *HRAS*, the over-expression of other candidate oncogenes, like *Ezh2*, *FoxM1* or *Bmi1* failed to induce tumor development. This work demonstrates that *Bmi1* over-expression leads to an increased neurosphere cell self-renewal and proliferation and a decrease in cell death. These BMI1 effects are relevant for the maintenance of tumor cells. How BMI1 exerts its functions remains only partly understood, and in this thesis, four novel BMI1 target genes are identified, which – based on their known functions - could contribute to the described BMI1 effects.

ZUSAMMENFASSUNG

Gehirntumore sind Neoplasmen des zentralen Nervensystems (ZNS) und werden nach ihrer Histopathologie klassifiziert. Aktuelle Therapien basieren auf dieser Einstufung, jedoch sind die genauen Faktoren die zur Ausprägung distinkter Gehirntumoren beitragen nicht bekannt. Die Aufklärung der molekularen Grundlagen sowie des zellulären Ursprungs von Gehirntumoren ist daher von grundlegender therapeutischer Relevanz. Die vorliegende Arbeit untersucht den Zusammenhang zwischen neuralen Stamm-/Vorläuferzellen und Gehirntumoren im Hinblick auf den zellulären Ursprung von Gehirntumoren und den Beitrag genetischer Veränderungen zu ihrer Entwicklung.

Im ersten Teil der vorliegenden Arbeit wurden Oberflächenmarker von neuralen Stamm-/Vorläuferzellen untersucht, um die Isolation und Untersuchung dieser Zellen zu ermöglichen. Der Oberflächenmarker CD133 wird in der Literatur als "neuraler Stammzellmarker" und potentieller phänotypischer Hinweis für eine Abstammung von Krebsstammzellen von ihren Ursprungszellen diskutiert. Die Präsenz von CD133 auf postnatalen Stammzellen des ZNS war zu Beginn dieser Arbeit jedoch nicht ausreichend geklärt. In dieser Arbeit wurden zwei CD133-positive Zelltypen in der postnatalen lateralen Ventrikelwand (LVW) identifiziert: post-mitotische Ependymzellen, die die Mehrheit der CD133-positiven Zellen der LVW darstellten, und eine Subpopulation von Ventrikel-kontaktierenden astrozytären Stammzellen. Zusätzlich wurden CD133-positive Ependymzellen im postnatalen Rückenmark (RM) untersucht. Im Gegensatz zu den CD133-positiven LVW-Ependymzellen, wiesen die Ependymzellen des RMs Stammzelleigenschaften, wie *in vitro* Selbsterneuerung und Multipotenz, auf. Eine vergleichende Genexpressionsanalyse beider Ependymzelltypen identifizierte mehrere in RM-Ependymzellen stärker exprimierte Gene, die – basierend auf ihre bekannte Funktion - von potentieller Relevanz für ihre Stammzelleigenschaften sein könnten. Darüber hinaus wurden in RM-Ependymzellen hochregulierte Gene gefunden, die in der Literatur als RM-Ependymoma-spezifische Signaturgene bekannt sind. RM-Ependymzellen wurden als potentielle Ursprungszellen von adulten RM-Ependymomen vorgeschlagen. Die hier beobachteten molekularen und funktionellen Gemeinsamkeiten zwischen sich selbst-erneuernden Stammzellen und Tumorzellen, deuten auf eine potentielle Abstammung der Gehirntumore von Stamm-/Vorläuferzellen hin.

Im zweiten Teil der Arbeit wurden zwei Fragen adressiert: 1) Welche onkogenen Faktoren initiieren die Entwicklung von Gehirntumoren aus neuralen Stamm-/Vorläuferzellen, und 2) Welchen Einfluss haben genetische Veränderungen bei der Spezifizierung des Tumorphänotyps? Diese Arbeit zeigte, dass die Kombination und Reihenfolge der *HRAS* und *MYC* Überexpression in *Trp53*-defizienten Zellen die Entstehung von drei distinkten Gehirntumortypen (Gliome, ZNS primitive neuroektodermale Tumore (PNETs) und atypische teratoid/rhabdoid (AT/RT)-artige Tumore) instruiert. In Genexpressionsprofilen der generierten murinen AT/RT-artigen Tumore sowie der humanen AT/RTs wurden Hinweise auf eine Aktivierung der „Unfolded Protein Response"(UPR) gefunden. Diese zelluläre Stressantwort wird unter bestimmten Konditionen wie Hypoxie oder Nährstoffmangel aktiviert und hat eine unterstützende Rolle für schnell wachsende Tumore wie AT/RTs. AT/RTs weisen typischerweise den funktionellen Verlust des Tumorsuppressors SMARCB1 auf. Untersuchungen in dieser Arbeit zeigen, dass eine reduzierte *SMARCB1* Expression zu einer erhöhten Sensitivität der eIF2a Phosphorylierung führt, was charakteristisch für eine aktivierte UPR ist. Basierend auf den Ergebnissen dieser Arbeit, wird die UPR als neuartiges Target für die Therapie von *SMARCB1*-defizienten Tumoren vorgeschlagen. Im Gegensatz zu *MYC* und *HRAS*, war die Überexpression von anderen Onkogenen, wie *Ezh2*, *FoxM1* oder *Bmi1* nicht ausreichend um eine

Tumorentstehung zu initiieren. Die vorliegende Arbeit zeigt, dass eine Überexpression von *Bmi1* in neuralen Stammzellen Selbsterneuerung und Proliferation fördert, sowie Zelltod unterdrückt. Diese Effekte sind von großer Relevanz für die Erhaltung von Tumorzellen, der genaue Mechanismus ist jedoch nur teilweise verstanden. In dieser Arbeit wurden vier neue BMI1-Targetgene identifiziert, deren bekannte Funktionen dazu beitragen die BMI1-vermittelten Effekte zu erklären.

INTRODUCTION

1) Human Brain Tumors

1.1 Classification and pathogenesis

Tumors are abnormal generations of tissue mass as a result of uncontrolled growth of cells. This aberrant form of cell proliferation is also termed neoplasia/neoplasms (Greek: new growth.)

Neoplasms that are well defined and restricted, not infiltrating other neighboring tissues are termed as benign. Many tumors are malignant – termed cancers – when they are characterized by certain hallmarks: they have the ability to grow in an uncontrolled and unlimited manner, are self-sufficient in growth signals and non-sensitive to anti-growth signals, able to evade normal cell death mechanisms (apoptosis), invade other tissues and form metastases and can also induce angiogenesis to support their rapid growth (Hanahan and Weinberg, 2000).

Benign and malignant neoplasms of the central nervous system are termed CNS or brain tumors. The yearly incidence of primary brain tumors is 7 per 100,000 individuals worldwide (Furnari et al., 2007; Ohgaki, 2009).These solid tumors show a wide range of intertumoral variety and are classified into CNS tumor entities by the World Health Organization (WHO) (Louis et al., 2007). Main criteria for this classification are distinct morphological appearance, location, age distribution and biology of the disease progression. The classification is oftentimes based on histological resemblance to cell types in the healthy brain tissue. Gliomas for example comprise all brain tumors with glial (-like) cell types including e.g. astrocytomas, oligodendrocyctomas, and ependymomas (Louis et al., 2007). They account for more than 70% of all primary CNS tumors (i.e. tumors primarily developing in the CNS in contrast to brain metastases from a tumor that developed in another organ)(Ohgaki and Kleihues, 2009).

With regards to disease pathology the WHO uses a grading system which indicates the severity of different CNS tumors (Louis et al., 2007). Grade I describes tumors with low cell proliferation, which grow non-infiltrative, are well circumscribed and can be cured by resection alone – one example is pilocytic astrocytoma, which is the most common brain tumor in children. Grade II brain tumors are more infiltrative and are likely to recur after surgery; however the cell proliferation potential is still rather low. Grade III tumors are malignant tumors with a high proliferative potential and characterized by nuclear atypia. Grade IV are the most aggressive forms of brain tumors; they are highly proliferative and invasive, with rapid disease progression and a fatal outcome. These tumors typically display necrosis and/ or microvasculature proliferation, indicative of blood vessel formation to support the rapid and aggressive tumor growth.

The following section describes three types of tumors, which are relevant for this thesis and belong to the most aggressive forms of brain tumors: Glioblastoma multiforme (GBMs), CNS primitive neuroectodermal tumors (CNS PNETs) and atypical teratoid rhabdoid tumors (AT/RTs). All three are WHO grade IV tumors.

1.1.1 Glioblastomas

The majority of gliomas occurring in adults are grade IV tumors (glioblastomas or "glioblastoma multiforme" - GBM). These astrocytic brain tumors have a very aggressive nature with fatal outcome for affected patients.

90% of GBMs develop *de novo* without any clinical history or histological indication of pre-existing, less malignant lesions and are designated "primary GBMs". Most of these arise in elderly patients with a median age of 62 years at time of diagnosis (Ohgaki and Kleihues, 2009). The remaining 10% are secondary GBMs which occur in patients with a median age of 45 and develop as a disease progression from less malignant forms of astrocytomas. For instance low grade diffuse astrocytomas (grade II) or anaplastic astrocytomas (grade III) can transform into GBM on average within 4.5 or 1.4 years respectively (Ohgaki and Kleihues, 2009). Despite advances in conventional therapies (surgery, radiotherapy, chemotherapy), the median patient survival in case of glioblastoma is still only 12-15 months (Stupp et al., 2005; Wen and Kesari, 2008).

High-grade malignant gliomas can also occur in children: 10% of all pediatric CNS tumors are anaplastic astrocytomas and GBMs (median age at diagnosis: 9-10 years); they occur mainly in the supratentorial cerebral cortex, the brainstem, and in the spinal cord. The overall prognosis for children is poor, but better than for adults. The overall survival rate in grade IV pediatric GBMs is 17% and long-term survival is reported for 20 to 30% of the affected children (Sposto et al., 1989; Finlay et al., 1995; Pollack et al., 2003).

Histological features of GBM include: Poorly differentiated anaplastic cells of astrocytic origin, and – as indicated by the term "multiforme" - cellular polymorphism. These tumors display a high mitotic activity, micro-vascular (endothelial cell) proliferation, and necrosis. The latter can occur as small pseudopalisading necrosis or as a large central necrotic area which takes up a great part of the tumor mass and is easily detectable by neuroimaging. Some cells have a high migratory potential resulting in an invasive phenotype and the formation of secondary tumor foci.

Primary and secondary adult and pediatric GBMs display largely indistinguishable histological and cellular features despite the differences in disease progression. However, the underlying genetic lesions identified in patients differ and suggest different modes of tumorigenesis (Maher et al., 2006; Tso et al., 2006; Furnari et al., 2007; Ohgaki and Kleihues, 2007)

1.1.1.1 Genetic lesions of adult primary and secondary GBMs: Loss of heterozygosity (LOH)

GBMs are characterized by a high grade of genetic instability and heterogeneity. The most frequent genetic alteration occurring in primary GBMs is the loss of heterozygosity (LOH) on chromosome 10 (> 70%); often the loss of a large chromosomal region (10q and 10p) can be observed (Karlbom et al., 1993; Rasheed et al., 1995; Ichimura et al., 1998; Fujisawa et al., 2000; Ohgaki et al., 2004). Three commonly deleted loci (10p14-15; 10q23-24 =PTEN; 10q25-pter) suggest the loss of several tumor suppressors as an important step for the pathogenesis of primary GBMs (Karlbom et al., 1993; Rasheed et al., 1995; Fults et al., 1998; Ichimura et al., 1998).

LOH at 10q is also frequent in secondary GBMs, although it is usually partial, and LOH of 10p is very rare in secondary GBMs (Fujisawa et al., 2000). The identified LOH at 10q25-qter could be associated with a progression from low-grade or anaplastic astrocytomas to GBMs.

LOH of chromosome 22q is found in 41% of primary, and 82% of secondary GBMs (Nakamura et al., 2005). The characterization of minimally deleted regions identified the *TIMP-3* locus (tissue inhibitor of metalloproteases), which is deleted in 22 of 23 secondary GBMs (Nakamura et al., 2005).

LOH of chromosome 13q occurs in 12% of primary and 38% of secondary GBMs (Nakamura et al., 2000). This typically includes deletion of the *RB* locus.

In contrast to malignant forms of oligodendroglioma, where the combined loss of chromosomes 1p and 19q can be observed (Cairncross et al., 1998; Bauman et al., 2000; Felsberg et al., 2004; Jenkins et al., 2006), LOH at 1p is infrequently found in both primary and secondary GBMs (12 and 15%; (Nakamura et al., 2000). However, LOH at 19q is frequent in secondary GBMs (54%) but rare (6%) in primary GBMs (Nakamura et al., 2000).

1.1.1.2 Genetic lesions of adult primary and secondary GBMs: Aberrant growth factor receptor signaling

The aberrant proliferation of tumor cells typically results from an intrinsic activation of growth signaling (see introduction below). Therefore it is not surprising to find genetic lesions that result in higher expression levels or activation of growth factor receptors.

Epidermal growth factor receptor (EGFR) amplifications are very rare in secondary GBMs but are frequent in primary GBMs (40%; Ekstrand et al., 1992; Watanabe et al., 1996; Ohgaki et al., 2004). Such amplifications always result in an over-expression of the *EGFR* (Tohma et al., 1998; Biernat et al., 2004). Additionally, *EGFR* amplifications are often associated with activating deletion mutants of the amplified gene. The most prominent is EFRvIII (deletion of exons 2-7). This mutated version of the EGFR has a mitogenic effect, as it leads to constitutive activation of the receptor and fails to attenuate receptor signaling by normal receptor down-regulation (Huang et al., 1997).

Additional genetic alterations which are found in primary GBMs but rarely in secondary GBMs are: *PTEN* mutations (15-40%; Tohma et al., 1998; Knobbe et al., 2002; Ohgaki et al., 2004), *NF1* mutations or homozygous deletions (18%), and loss of *PIK3R1* (10%; TCGA, 2008).

The over-expression of another growth factor, namely *PDGFR*, is found on a variety of astrocytic tumors of all grades. The encoding gene is typically over-expressed in secondary GBMs, however, gene amplifications are rarely found or are restricted to subsets of primary GBMs (Hermanson et al., 1992; Maher et al., 2006; 2008)

1.1.1.3 Genetic lesions of adult primary and secondary GBMs: Impaired tumor suppressor functions

P53 is one of the best characterized tumor suppressors, which has a functional role in apoptosis signaling, response to DNA damage, differentiation, and negative regulation of the cell cycle (Bogler et al., 1995). Activated p53 (encoded by *TP53*) induces transcription of p21 (*CDKN1A*), which leads to a negative regulation of the cell cycle. Negative regulators of p53 are MDM4 and MDM2, which can inhibit its transcriptional activity. MDM2 itself is repressed by one gene product of the *CDKN2A* locus: $p14^{ARF}$ which thereby derepresses p53 function.

Overall, a study by Zawlik et al. (2009) showed that >70% of secondary GBMs and about 50% of primary GBMs display at least one alteration leading to a p53/MDM2/p14 pathway inactivation. *TP53* mutations are more frequent in secondary GMBs than in primary GBMs (65% vs 28%; Ohgaki et al., 2004; Ohgaki and Kleihues, 2005). In addition, the majority of *TP53* mutations in secondary GBMs occur in certain hot spot codons (248 and 273), whereas *TP53* mutations in primary GBMs are spread randomly. There is a significant inverse correlation between *MDM2* amplifications and *TP53* mutations, as well as between *P14ARF* alterations and *TP53* mutations: Amplification of *MDM2* occurs rather infrequently (15%) and exclusively in primary GBMs without *TP53* mutations. Loss of p14 activity by promoter methylation is frequent in primary and secondary GBMs (Nakamura et al., 2001), and deletions of *CDKN2A* and *TP53* occur as mutually exclusive events (Fulci et al., 2000).

A study of The Cancer Genome Atlas Research Network (TCGA, 2008) showed a high frequency of genetic alteration of the *TP53/MDM2/P14ARF* axis (87%) in the analyzed GBM samples (predominantly primary GBMs), namely *TP53* mutations or homozygous deletions (35%), amplifications of *MDM2* (14%) or *MDM4* (7%), or *P14ARF* homozygous deletions or mutations (49%).

Another axis of tumor suppression and cell cycle regulation is the RB pathway: RB1 binds to E2F transcription factors, thereby preventing the progression through the cell cycle. Cell cycle kinase CDK4/cyclinD1 complexes release E2Fs from this binding by phosphorylating RB1. This results in G1-S transition and cell cycle progression.

The RB pathway function could be impaired due to either deletion of *P16INK4A* (an activator of RB signaling) or *RB1*, or by the amplification of *CDK4*. These three genetic events were found to be mutually exclusive, but overall genetic alterations, which affect either of these, occur at a frequency of 50% in primary and 40% in secondary GBMs. TCGA (2008) found a high frequency of genetic alterations in primary GBMs – most of them caused by a homozygous deletions of *P16INK4A* (52%), *P15INK4B* (47%) and less frequent by deletions of *P18INK4C* (2%) or deletions and mutations of *RB1* (1%). *CDK4*, *CDK6* or *cyclinD2* were found amplified in 18%, 1% and 2% of the cases, respectively.

A newly discovered genetic alteration, not until recently associated with gliomas are mutations of the *IDH1* locus. *IDH1* encodes the isocitrate dehydrogenase 1, which catalyzes the oxidative carboxylation of isocitrate to alpha-ketoglutarate in the Krebs (citric acid) cycle. Heterozygous mutations result in a dominant negative effect on wild-type IDH1 activity, as catalytically inactive heterodimers are formed. *IDH1* mutations were found frequently (80%) in low-grade astrocytomas, anaplastic astrocyctomas and secondary GBMs, as well as in oligodendrogliomas, astrocytic oligendrogliomas and anaplastic oligoastrocytomas (Balss et al., 2008; Watanabe et al., 2009; Yan et al., 2009). In contrast, mutated *IDH1* is rarely found in pilocytic astrocytomas and primary GBMs.

To summarize the genetic lesions in adult malignant gliomas: Primary GBMs, which arise *de novo,* can be genetically characterized by loss of heterozygosity on chromosome 10q, *EGFR* amplifications, deletions and mutations of the gene loci *CDKN2A* and *PTEN*. In contrast, secondary GBMs typically display a higher frequency of *TP53* and *IDH1* mutations and a loss of heterozygosity on chromosome 19q.

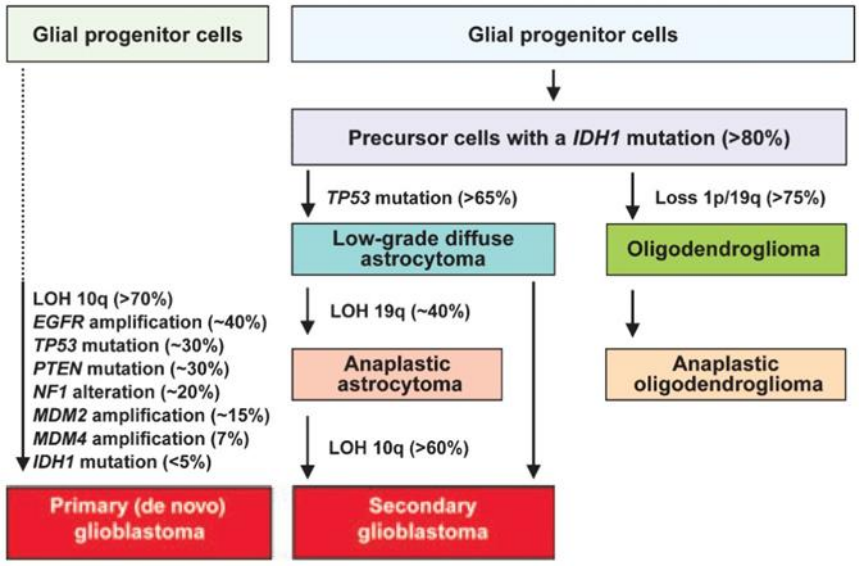

Fig. 1: Characteristic genetic alterations in primary and secondary GBMs. Figure taken from Ohgaki and Kleihues (2009).

1.1.1.4 Genetic alterations in high-grade pediatric gliomas

Although high-grade malignant gliomas in children and adults are histologically very similar, the underlying genetic lesions display some differences. *EGFR* amplifications are among the most frequent genetic alterations in primary GBMs and are often accompanied by the loss of chromosome 10 with a deletion or mutation of the *PTEN* locus. These alterations appear less commonly in children (Bredel et al., 1999). However, the fewer cases of *PTEN* deletions or mutations, which are observed in pediatric GBMs, are generally associated with a worse prognosis (Ullrich and Pomeroy, 2006)

TP53 mutations occur in 40% of childhood malignant gliomas, comparable to the frequency observed in adult malignant glioma. Especially in secondary GBMs, inactivation of *TP53* appears to be an important prerequisite for the malignant disease progression from lower-grade gliomas. However, while such a progression is not typically observed in pediatric glioblastomas, Pollack et al. observed a worse prognosis in children with *TP53* mutations compared to those without (Pollack et al., 1997).

Molecular abnormalities resulting in the dysregulation of cell cycle control genes (deletion of *RB*, *CDKN2A* (p19, p14), or amplification of *CDK4*) are common in both, pediatric and adult GBMs (Biegel and Pollack, 2004).

1.1.2 CNS primitive neuroectodermal tumors (CNS PNETs)

CNS PNETs belong to the group of embryonal tumors (as well as medulloblastomas and AT/RTs; Louis et al., 2007). Due to their location, CNS PNETs were previously termed supratentorial PNETs, to differentiate them from infratentorial PNETs, synonymously used for medulloblastomas. To date, CNS PNETs designate poorly differentiated tumors occurring at any extracerebellar site of the CNS (Louis et al., 2007). They predominantly occur in children (with a mean age of 5 to 7 years), are quite

rare and are very seldomly found in adults (Brandes et al., 2009). In contrast to medulloblastomas, CNS PNETS are more aggressive, resulting in a 5-year survival of only 20-30% of affected patients.

Very similar to medulloblastomas they are composed of densely packed, primitive undifferentiated neuroepithelial cells of small round to carrot shaped morphology, which contain hyperchromatic nuclei. The tumors can show a divergent differentiation potential along the neuronal, glial, or rarely mesenchymal lineage and can therefore show positive staining for synaptophysin, GFAP and neurofilaments in immunohistological analyses.

CNS PNETs are cytologically and morphologically difficult to distinguish from medulloblastomas, however these two tumor types differ in their genetic alterations.

For instance, *PTEN*, which is found to be lost in 30% of medulloblastomas, is never deleted in CNS PNETs. Although MYC seems to play an important role for both types of embryonal tumors, amplifications of *MYCN* are not as frequently found in CNS PNETS (30%) as in medulloblastomas (60%). On the other hand, deletion of *CDKN2A* was found to be a characteristic genomic alteration of CNS PNETs (30%; Kagawa et al., 2006).

1.1.3 Atypical teratoid/rhabdoid tumors (AT/RTs)

AT/RTs are aggressive embryonal tumors, which were defined as an entity by Rorke et al. (1996). Although CNS rhabdoid tumors or embryonal CNS tumors occuring in infants in association with malignant rhabdoid tumors of the kidney have been documented since 1985, they were previously misdiagnosed as PNETs or medulloblastomas (Rorke et al., 1996).

They are most frequently diagnosed in young children (<4 years old). The very aggressive nature of AT/RTs is displayed by a median time of disease progression of only 6-7.5 months with fatal outcome for the patients. However, long-term survivors have been reported (Biegel and Pollack, 2004; Hilden et al., 2004).

AT/RTs can be found in different region of the brain: 33% of the AT/RTs are located in the cerebellar parenchyma, 25% in the cerebellum, 20% in the cerebello-pontine angle and 14% in the pineal. (Biegel and Pollack, 2004). Apart from their histologically typical appearance with sheets of rhabdoid cells, AT/RTs can also display histological features typical for PNETs, but can also contain mesenchymal components with spindle like cells. Typical rhabdoid cells have an enlarged, vesicular, eccentric nucleus containing a prominent nucleolus. The cell body may be large and plump, or spindle-shaped, with homogenous appearance or prominent hyaline cytoplasmic inclusions. Three epitopes are almost always present in AT/RTs and can be detected by immunohistochemical analyses: Epithelial membrane antigen (EMA), vimentin and smooth muscle actin. Another important feature is the lack of *SMARCB1* (INI1/SNF5/BAF47) immunoreactivity. Approximately 70% of AT/RTs display loss of both copies of *SMARCB1* or loss of one allele and subsequent mutation of the *SMARCB1* gene on the remaining chromosome. 15 to 25% of rhabdoid tumors have other types of genetic alterations, which secondarily results in a loss of *SMARCB1* expression at the mRNA or protein level (Biegel et al., 2002).

SMARCB1/INI1/SNF5 is a component of the SWI/SNF chromatin remodeling complex, which has a function in regulation of gene expression. *SMARCB1* has been implicated to act as a tumor

suppressor gene in malignant rhabdoid tumors (Klochendler-Yeivin et al., 2000; Biegel and Pollack, 2004). Germline mutations in *SMARCB1* were described as the first of a "two-hit" mechanism of tumorigenesis and predispose infants to the development of renal and extrarenal malignant rhabdoid tumors as well as to AT/RTs and other CNS tumors (choroid plexus cell carcinoma, medulloblastoma and PNET; Biegel et al., 1999; Sevenet et al., 1999; Janson et al., 2006). Sevenet et al. (1999) proposed the term "Rhabdoid predisposition syndrome" for these cases with germline constitutional *SMARCB1* mutations. J. Biegel's laboratory compared several familial cases of CNS AT/RTs (with germline/hereditary predisposition) to sporadic AT/RTs (Bruggers et al., 2010). They reported that familial AT/RTs occur in younger children (median age at diagnosis: 13 in sporadic, 4.8 months in familial AT/RTs) and are more likely to have a fatal outcome for the patients (median survival of 21 months in sporadic vs 4.5 months in familial AT/RTs).

1.2 Origin and identity of human brain tumors

To pinpoint the normal cell of origin and the molecular event(s) leading to the development of malignant brain tumors has been and still is a challenge. The phenotypic tumor resemblance to normal cell types in the healthy brain or the location can only give vague hints as to where in the brain the tumor originates from. A clinical study on the location of GBMs only modestly implied that the tumors could be derived from a stem cell region in the healthy brain (Lim et al., 2007). Due to low spatial and temporal resolution of current imaging modalities, as well as the highly invasive potential of malignant glioma cells, it has not yet been possible to show a direct tumor progression from suggested stem/progenitors cells of the lateral ventricle.

Therefore mouse models are needed and were already successfully applied in the past to address the following two fundamental questions: What is the cellular origin of brain tumor cells, and in addition: which genetic/molecular event causes normal brain tissue to develop into brain tumors (reviewed in (Visvader, 2011; see introduction below). These questions can be extended with regards to causal effects: Do different subtypes of brain tumors originate from different cell types or is the intertumoral heterogeneity rather due to different genetic alterations in a common similar or identical cell of origin. Investigations addressing these questions are highly relevant to reveal the malignant potential of human neural stem cells to initiate tumor growth. As the identification of new markers for the progression of brain tumors would already assist an earlier cancer detection, identifying the source of human brain tumors and truly understanding the molecular factors of brain tumor formation may lead to an improved therapeutic targeting and the development of new therapeutic drugs. In addition, a profound knowledge of the identity and function of normal neural stem/progenitor cell types is crucial to properly understand their potential relationship to brain tumor cells.

2) Stem and progenitor cells in the murine central nervous system

The two hallmarks of stem cells are the ability to self-renew for an indefinite period of time and to give rise to specialized cell types of the tissue or organ they are derived from. Self-renewal divisions can be symmetric, generating two identical daughter stem cells, or asymmetric, which results in a stem cell and a further differentiated cell (Potten and Loeffler, 1990).

2.1 Neural stem and progenitor cells in the developing forebrain

The first neural stem cells during development are neuroepithelial cells, which compose the wall of the neural tube (Merkle and Alvarez-Buylla, 2006). Around embryonic day 9-10 (E9-10), at the onset of neurogenesis, neuroepithelial cells are replaced by radial glial cells (RGCs; Gotz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009). RGCs have long radial processes extending to the pial, while their cell body remains in the ventricular zone (Fig.2). RGCs and neuroepithelial cells share the expression of Nestin, however only RGCs synthesize proteins characteristic for 'glial' cells, such as the Glutamate/aspartate transporter (GLAST), Brain lipid binding protein (BLBP), S100 and Vimentin (Mori et al., 2005). RGCs mainly divide asymmetrically to self-renew and generate a further differentiated daughter cell and constitute a heterogeneous cell population: Uni- and multipotent RGCs exist and dependent on location and time, RGCs can give rise to different subtypes of neuronal or glial cells (Mori et al., 2005; Kriegstein and Alvarez-Buylla, 2009).

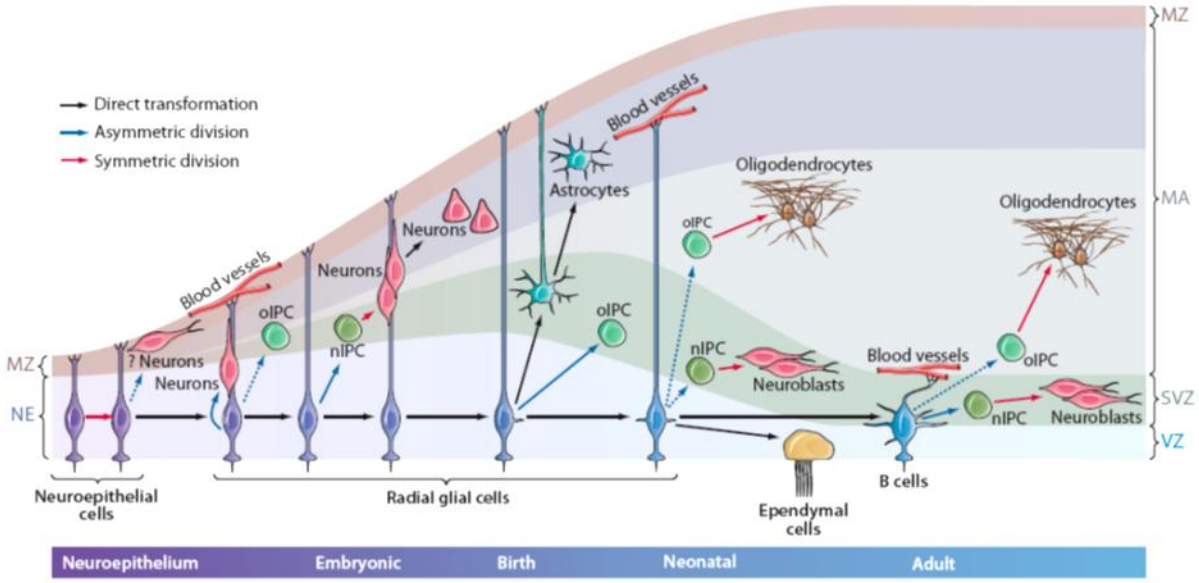

Fig. 2: Neural stem cells and their progeny in the developing and adult murine brain. Ventricle lumen: bottom part of figure, pial surface: top part of figure, solid arrows: experimental evidence, dashed arrows: hypothetical connections. MA, mantle; MZ, marginal zone; NE, neuroepithelium; nIPC, neurogenic intermediate progenitor cell; oIPC, oligodendrocytic intermediate progenitor cell; SVZ, subventricular zone; VZ, ventricular zone. Figure taken from Kriegstein and Alvarez-Buylla (2009).

RGCs can also generate intermediate progenitor cells (IPCs), which are located in the region above the ventricular zone, the so-called subventricular zone (SVZ, Fig.2). Neuronal IPCs divide symmetrically to produce two neurons or two new IPCs, thereby forming a secondary proliferative layer and amplifying the number of generated neurons. IPCs generating oligodendrocytes and potentially astrocytes exist as well.

RGCs disappear within the first two weeks after birth, when most RGCs disconnect from the ventricle, migrate to the cortical plate and convert into postmitotic glial cells (Mori et al., 2005; Kriegstein and Alvarez-Buylla, 2009). Alvarez-Buylla and colleagues showed that RGCs also give rise to neurogenic astrocytes (B cells) and ependymal cells (Fig.2) in the lateral ventricle wall of the adult brain (Merkle et al., 2004; Spassky et al., 2005).

2.2 Neurogenesis in the adult forebrain

The lateral ventricle wall (LVW) region is the largest neurogenic zone in the adult rodent brain (Alvarez-Buylla and Garcia-Verdugo, 2002). LVW stem cells constantly produce new neurons, which migrate along a defined route, the rostral migratory stream (RMS), to the olfactory bulb where they differentiate into periglomerular and granule interneurons (Alvarez-Buylla and Garcia-Verdugo, 2002).

The LVW consists of four major cell types: Ependymal cells (type E cells), neuronal precursors (neuroblasts; type A cells), type B cells and transit-amplifying type C cells. Type B cells are positive for glial fibrillar acidic protein (GFAP). This cell population can be further subdivided based on function and morphology (Doetsch et al., 1997; Mirzadeh et al., 2008; Shen et al., 2008). A subfraction of type B cells, whose cell bodies are either in close proximity to or intercalated between ependymal cells, contacts the ventricle via an apical processes and have long basal processes which terminate on blood vessels (Fig.3). It is not known yet, whether all or only a fraction of these ventricle-contacting type B cells carry the surface protein CD133 at their primary cilium and apical surface (Beckervordersandforth et al.; Mirzadeh et al., 2008). Type E cells form a continuous layer of cells along the lateral ventricle wall and have long motile cilia at their apical membrane (Fig.3). Two morphological different type E cells are present in the adult LVW: Multiciliated type E1 and biciliated type E2 cells (Mirzadeh et al., 2008). Ependymal cells are, at least in part, derived from RGCs (see above) and the transition from RGCs to ependymal cells occurs in the first postnatal week via an intermediate stage, where the cells co-express RGC proteins, such as GLAST and a protein of mature LVW ependymal cells, e.g. S100 (Spassky et al., 2005). Mature ependymal cells (type E1 and E2) can be identified by their markers CD133, CD24 and S100, and they are negative for GFAP (Mirzadeh et al., 2008).

The identity of the LVW stem cell has been a matter of debate. Johansson et al. (1999) suggested that ependymal cells are the LVW neural stem cells, but these findings were challenged shortly after by Doetsch et al. (1999b), who provided evidence that type B cells are the neural stem cells in the ventricular wall (Doetsch et al., 1999b; Doetsch et al., 1999a; Johansson et al., 1999). The latter findings were supported by subsequent studies (Chojnacki et al., 2009) and led to the current, commonly accepted model: Self-renewing type B stem cells give rise to transit-amplifying type C cells, which in turn generate type A cells. Morphologically different subpopulations of type B cells were identified but their functional properties are not yet fully elucidated (Chojnacki et al., 2009).

However, it was shown by different groups that CD133-positive type B cells and ventricle-contacting type B cells are neurogenic *in vivo* and *in vitro* (Beckervordersandforth et al.; Mirzadeh et al., 2008).

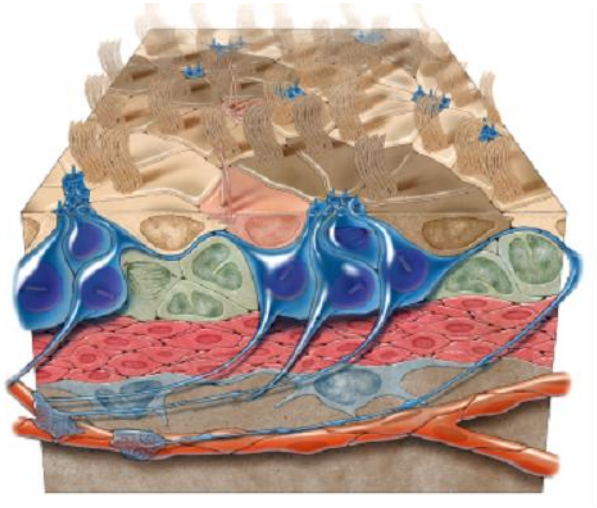

Fig. 3: Model of the LVW neurogenic region. Figure taken from Alvarez-Buylla and Garcia-Verdugo (2002).

Controversial findings were published about the functional properties of ependymal cells in the adult LVW. It was suggested by two studies that LVW ependymal cells are (quiescent) stem cells (Johansson et al., 1999; Coskun et al., 2008) whereas others found them to be postmitotic cells (Spassky et al., 2005; Mirzadeh et al., 2008). A recent publication by Jonas Frisen's group resolved some of these controversies and provided convincing evidence that LVW ependymal cells do not fulfill the defining criteria of stem cells. Using genetic fate mapping, this study revealed that LVW ependymal cells do not divide or give rise to progeny under steady-state conditions. However, pathological conditions, e.g. stroke, could activate these cells to enter the cell cycle and differentiate into neuronal cells or astrocytes. However, the generation of progeny resulted in the loss of the ependymal cells, indicating that these cells are not able to self-renew (Carlen et al., 2009).

2.3 Neural stem and progenitor cells in the postnatal spinal cord

Early *in vitro* experiments by Weiss et al. (1996) showed that the adult SC contains cells with neural stem/progenitor cell properties, however their exact location and identity remained unclear. Neural stem/progenitor cells were suggested to reside in the area around the central canal, in the parenchyma of the SC, or in both regions (Barnabe-Heider and Frisen, 2008; Obermair et al., 2008) and different cell types were proposed: ependymal cells, oligodendrocyte progenitor cells and astrocytes (Barnabe-Heider et al., 2010 and references therein).

Ependymal cells, which were suggested to originate from NKX6-1 positive ventral neuroepithelial cells (Fu et al., 2003), are located around the central canal of the SC. They have one to three motile cilia, synthesize CD133 and can be subdivided into morphologically different subpopulations (Meletis et al., 2008). Already in 1999, ependymal cells were proposed as spinal cord stem cells by Frisén and colleagues (Johansson et al., 1999). However, the techniques used in this and following studies did

not allow to determine whether ependymal cells or other cells in close proximity to the central canal are the stem cells in this region. Recent publications which specifically labeled adult spinal cord ependymal cells and their progeny by a cre-loxP fate mapping technique confirmed the ependymal identity of SC stem cells (Meletis et al., 2008; Barnabe-Heider et al., 2010). Under steady-state conditions, ependymal cells are quiescent *in vivo*, i.e. they do not give rise to progeny and divide only rarely for self-renewal purposes. Upon injury however, they start to proliferate extensively in order to self-renew and generate progeny along the astrocytic and oligodendrocytic lineage (Meletis et al., 2008; Barnabe-Heider et al., 2010).

Other cells, such as oligodendrocyte progenitor cells and astrocytes, contribute to the steady-state cell maintenance and the response to injury. Genetic fate mapping showed that both populations are able to self-renew, but can only give rise to their own kind under physiological and pathological conditions (Barnabe-Heider et al., 2010).

2.4 The cancer stem cell concept

Cells with stem cell properties have also been described in tumors. Analogous to stem cells, a cancer stem cell is defined as a cell which is able to self-renew and give rise to all the cell types of the tumor. For human brain tumors, the concept of cancer or tumor stem cells has been established by the isolation of a subpopulation of brain tumor cells, which could be identified and isolated based on their surface protein CD133. Experiments by the Peter Dirks' group indicate that BTSCs have stem cell properties like self-renewal and multipotency *in vitro* (Singh et al., 2003). Long term *in vivo* self-renewal could be demonstrated by serial xenotransplantation of as few as 100 of the CD133 positive cells recapitulating the original tumor growth in a NOD-SCID mouse model (Singh et al., 2004). This concept is of clinical relevance, as it implies that anti-tumor therapies need to specifically target BTSC, especially as it has been suggested that BTSCs are more resistant to chemo- and radiotherapy (Bao et al., 2006; Liu et al., 2006).

The strategy of using one or a combination of surface markers to enrich for cancer stem cell subpopulations has been applied to a variety of tumors. CD133 gained increased attention since it was found as a tool to enrich cancer stem cells in a variety of other human tumor tissues, such as prostate tumors, pancreatic adenocarcinomas, colon and hepatocellular carcinomas and renal tumors (Collins et al., 2005; Bruno et al., 2006; Suetsugu et al., 2006; O'Brien et al., 2007; Olempska et al., 2007; Ricci-Vitiani et al., 2007). In addition CD133 was also found to be present on normal stem and progenitor cell types of several of the corresponding tissues (Richardson et al., 2004; Bussolati et al., 2005; Koblas et al., 2007). A similar situation was found for brain tumors and neural stem cells: 1) The phenotypic marker CD133 used to identify brain tumor initiating cells is also found on normal neural cell types with stem cell features (Weigmann et al., 1997; Uchida et al., 2000; Lee et al., 2005). 2) Isolated CSCs from primary brain human tumors share neural stem cell features like *in vitro* self-renewal and multipotent differentiation capacity (Singh et al., 2003). However, the conclusion, that human tumor stem cells are directly derived from normal human stem cells has yet to be proven and is not part of the CSC definition.

3) Molecular basis for neural stem/progenitor cell and brain tumor cell function

Neural stem cells and brain tumor stem cells obviously share key features, like the capacity for long-term self-renewal. Accumulating evidence indicates that similar molecular programs, which involve proto-oncogenes and tumor suppressor genes, regulate normal stem cell and tumor stem cell self-renewal (Pardal et al., 2005). These programs or pathways include molecular factors that:

1) Keep normal stem cells in an undifferentiated state by restricting differentiation. These programs are responsible for maintaining the stem cell identity and function.

2) Control the cell cycle. This enables the controlled and limited proliferation of stem cells in response to growth factor signaling.

While those pathways are strictly regulated in normal stem cells according to physiological demand, they are dysregulated in tumor stem cells, resulting in poorly controlled self-renewal.

3.1 Self-renewal and mitogenic cues in normal and brain tumor cells

In normal cells proliferation requires extracellular mitogenic signaling via diffuse growth factor binding, the interaction with the extracellular matrix (ECM), and cell-cell interactions. Receptors that sense these kinds of interactions activate a downstream repertoire of intracellular signaling pathways, which may activate cell cycle genes and induce proliferation. Tumor cells acquire mutations that reduce or even eliminate the dependence on extracellular signal stimulation. This equips them with a constitutive activation of proliferation-inducing pathways, evading the need for extracellular growth stimulation. Mutations that lead to the constitutive activation of such receptors are commonly found in gliomas.

3.1.1 The MAPK/ERK pathway

The mitogenic MAPK/ERK (microtubule-associated protein kinase/extracellular signal regulated kinase) pathway includes signaling via growth factor receptors which are receptor tyrosine kinases (RTK). RTKs are activated by ligand binding, which usually induces receptor dimerization and autophosphorylation. They bind and activate adaptor proteins and GTP exchange factors, which signal to the small GTPases of the RAS superfamily. Activated RAS proteins then transduce the signal via a phosphorylation signaling cascade of mitogen activated kinases (RAF, MEK and MAPK). MAPK eventually activates a series of transcription factors (e.g. MYC) which induces the expression of cell cycle genes. This pathway is essential for proliferative cells. Normal neural stem cells in the LVW express RTKs that activate MAPK pathways, for example the epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor (PDGFR).

Kuhn et al. (1997) could demonstrate that the rat SVZ expands due to proliferation in response to EGF administration. They reported the formation of small polyp-like hyperplastic structures from the subventricular zone into the ventricles, however this proliferative effect was reverted to normal, when EGF administration was ceased.

The group of A. Alvarez-Buylla has shown the presence of the epidermal growth factor receptor on the majority of transit amplifying progenitor cells (type C cells) and a subset of neurogenic astrocytes (type B cells), which contacted the lateral ventricle in the adult murine SVZ (Doetsch et al., 2002). This indicates that EGFR signaling is important for both, highly proliferative neural progenitor cells (type C) and undifferentiated, slowly cycling type B stem cells of this region.

Jackson et al. demonstrated that the ventricle contacting neural stem cell in the murine SVZ also express another RTK: the platelet-derived growth factor alpha (PDGFRa) (Jackson et al., 2006).

The deregulation of mitogenic signaling in malignant glioma cells can arise at different levels within the MAPK pathway. As described above, RTK activation by *EGFR* amplification/ activating mutations and *PDGFR* over-expression are found at high frequencies in human adult GBMs. The central role of overactive EGFR signaling for gliomagenesis was also shown in mouse models, where the *EGFRvIII* activity in combination with a tumor suppressor was sufficient to transform cultured normal neural stem/progenitor cells or astrocytes into glioma-forming cells (Bachoo et al., 2002; Bruggeman et al., 2007).

Additionally, high levels of the GTPase RAS are frequently found in malignant gliomas (Guha et al., 1997). This might result primarily from the high activity of upstream RTKs (constitutively active *EGFRvIII* or over-expressed *PDGFR*), or from mutations or homozygous deletions of the negative regulator *NF1* (TCGA, 2008), as *HRAS* mutations or amplifications are only rarely detected (Knobbe et al., 2004).

3.1.2 PI3K/AKT signaling

Another downstream pathway of RTK signaling in normal and glioma cells is the PI3K/AKT pathway. RTKs and RAS activate phospho-inositol-3-kinases (PI3K), which form heterodimers and catalyze the phosphorylation of phosphatidylinositols(4,5)P2 (PIP2) to phosphatidylinositols(3,4,5)P3 (PIP3). PTEN is an antagonist of PI3Ks functions, as it dephosphorylates PIP3 to PIP2.

PIP3 subsequently recruits AKT (also known as protein kinase B) to the inner cell membrane, where it gets activated by PDK. Activated AKT has a variety of downstream targets which contribute to the inhibition of apoptosis (by repressing BAD and stimulating NFkB) and stimulate growth and cell cycle progression (e.g. by via mTOR and GSK3b). Deregulation of the AKT pathway is considered a common feature in gliomagenesis. PTEN which inhibits PI3K function at an early step in this mitogenic pathway, is found mutated or deleted (by loss of chromosome 10q) in up to 40% of all primary glioblastomas (Ohgaki and Kleihues, 2009). In addition, the amplification of *AKT3* and members of the PI3K family could be detected in a subset of glioblastomas (TCGA, 2008).

In normal neural stem cells *PTEN* has a role in restricting stem and progenitor cell proliferation. Deletion of *PTEN* in the CNS results in increased *in vitro* and *in vivo* self-renewal and proliferation capacity of murine fetal neural stem cells (Groszer et al., 2001) and a decreased growth factor dependency without affecting multipotency (Gregorian et al., 2009). Adult type B LVW stem cells with *PTEN* deletions displayed increased *in vivo* proliferation, resulting in an overall expanded number of stem/progenitor cells and their progeny. However, the *PTEN* deletion does not result in tumorigenesis or premature senescence, and does not interfere with endogenous migration and differentiation behavior of cell generated from the affected type B cells (Gregorian et al., 2009).

With regards to therapy, overactive EGFR and PDGR activity seems to be the driving force for aberrant ERK and PI3K/AKT signaling, suggesting the use of EGFR and PDFR inhibitors to counter glioma growth. However, Stommel et al. (2007) report that conventional therapies targeting single RTKs results in only modest improvements of outcome in GBM patients. Furthermore they found that multiple RTKs are co-activated in these tumors and demonstrated that only the combined use of several different RTK inhibitors resulted in the abrogation of PI3K signaling, decreased cell survival, and growth inhibition of glioma cells in culture.

3.1.3 Notch signaling

Notch signaling represents another pathway with similar functional importance for neural stem cells and glioma cells. Notch is a transmembrane protein; it gets activated by binding to its ligands of the Delta and Jagged family which leads to cleavage of Notch, resulting in a truncated intracellular domain of the protein (NIC). NIC translocates into the nucleus, where it activates the transcription of a family of bHLH transcription factors known as hairy/enhancer of split (HES) genes. Two of these, *Hes* and *Herp* (Hes regulated protein) are most commonly activated and known to antagonize proneural differentiation. Interestingly Numb/Numbl proteins have generally been suggested as negative regulators of Notch signaling.

Numb proteins display an asymmetrical distribution during cell division, and can therefore inhibit Notch in one of the daughter cells. *In vitro* and *in vivo* loss-of function analyses have suggested that asymmetric Numb segregation determines cell identity in the nervous system (Fishell and Kriegstein, 2003). However, there is contradictive data regarding the function of Numb/Numb-like proteins – some knock-out studies indicate that they promote differentiation, others imply that Numb/Numb-like are maintaining the progenitor pool (reviewed in Yoon and Gaiano, 2005; Pierfelice et al., 2008).

Notch itself has been reported to confer radial glial cell identity and is implicated in the restriction of neural differentiation in radial glial cells during development (Gaiano et al., 2000). The brain-lipid-binding protein, a marker for RGCs is a direct target of Notch signaling. Over-expression of Notch receptors and ligands has been observed in glioblastomas (Ignatova et al., 2002; Purow et al., 2005), and inhibition of the Notch pathway by siRNAs directed against *Notch1*, and its ligands *Dll1* and *Jag1* leads to decreased proliferation of glioma cell lines *in vitro*, and prolonged survival in an orthotopic brain tumor model (Purow et al., 2005). Kanamori et al. (2007) showed similarly, that the application of a gamma-secretase inhibitor (preventing the cleavage of the Notch receptors and following NIC translocation to the nucleus) represses the growth of glioma cell lines. In addition, expression levels of the Notch pathway target *Hey1* were found to correlate with increased grade in malignant astrocytomas, whereas its repression by means of RNA interference leads to decreased proliferation of GBM cell *in vitro* (Hulleman et al., 2009).

3.2 Function of tumor suppressor regulation for neural stem cells and brain tumor cells

Mitogenic and self-renewal pathways mentioned above enable normal stem cells properties, which are important for tissue regeneration. Mutations, which result in constitutive activation of key components of these pathways (proto-oncogenes) impose the danger of neoplastic transformation

upon stem cells, however the protective function of tumor suppressor genes needs to be overcome (Green and Evan, 2002).

Tumor suppressors are generally divided into caretakers and gatekeepers (Pardal et al., 2005). Caretakers have an imminent role in preventing mutations and thus the accumulation of potentially oncogenic events – e.g.: *ATM* (ataxia telangiectasia mutated), which has a role in detecting and promoting repair of DNA damage. Gatekeepers (e.g. *RB* or *TP53*) play a critical role in restricting cell proliferation in case cells become mutated or stressed. When required, gatekeepers are able to initiate cell death or senescence – which is a specialized form of terminal differentiation, rendering the cell irreversibly post-mitotic.

A normal function of tumor suppressors is of particular importance in stem and progenitor cells. Mutation in stem cells could be carried over to the large number of progeny, increasing the risk of accumulating mutations required for oncogenic transformation. In contrast to caretakers, the mode of action of gatekeepers - required to counter over-activity of stem cell properties like self-renewal/proliferation - also sets limits to stem cells' function in tissue regeneration. This is exemplified by the expression levels of some gatekeeper tumor suppressor genes like *CDKN2A* (encoding p16 and p19), which are very lowly expressed during development (when rapid tissue growth is needed) but display increased expression levels with ageing (Pardal et al., 2005). This suggests that gatekeepers - by restricting stem/cells progenitors - participate in the age-related decline of tissue regeneration.

In conclusion, normal stem cells require the coordinated balance of proto-oncogenes (which can promote self-renewal but also neoplastic transformation) and tumor suppressors (which inhibit oncogenesis but also restrict regeneration; Pardal et al., 2005).

3.2.1 Retinoblastoma (RB)

RB (retinoblastoma) is the first "classical" human tumor suppressor gene, described by Knudson et al. (1971) following the "two-hit" model of tumorigenesis. The majority of tumor suppressor genes can be defined as genes with cancer preventive effect, which require the inactivation of both their alleles to allow for a contribution to tumorigenic transformation (Sherr, 2004). In case of the *RB* tumor suppressor gene, retinoblastoma development via mutation or loss of a second intact allele is reported for 95% of the individuals who received a germline transmitted *RB* mutation (Gallie et al., 1990). However, some tumor suppressors function as haploinsufficient factors, i.e. heterozygous mutations are already sufficient to promote tumorigenic transformation (Sherr, 2004).

The originally identified retinoblastoma gene actually belongs to a family of RB proteins, which have a fundamental function for the progression of the cell cycle: Active hypophosphorylated RB constantly binds to and inhibits family members of E2F transcription factors (Sherr and McCormick, 2002). Mitogenic signaling stimulates CDKs (cyclin dependent kinases) which get activated by binding to their appropriate cyclin partners. CDK/cyclin complexes repress RB proteins by phosphorylation. Subsequently, E2Fs are released from RB binding and activate the transcription of genes important for S-Phase entry.

In GBMs loss of *RB1* could only infrequently be observed (TCGA, 2008). Impairment of the RB signaling is still an important step in gliomagenesis, but predominantly occurs via loss of the CDK-inhibitor *P16INK4a*.

3.2.2 p53 signaling

TP53 (tumor protein p53 in human/ Trp53 - transformation related protein 53 in mouse) is generally considered the most important tumor suppressor gene. It is found inactivated in >50% of all tumors (Sherr, 2004). The recognition that *TP53* is the most singly most frequently inactivated gene in all human cancers underlines its central role as a tumor suppressor and the importance of p53-mediated cell cycle arrest (Olivier et al., 2002). *TP53* mutations have been found to be the causative genetic factor underlying the LiFraumeni syndrome, a familial disease, in which patients display an increased susceptibility to develop a variety of different cancers at relatively young age (<45 years) (Malkin et al., 1990).

The *TP53* encoded protein acts as a homotetrameric transcription factor, which is activated in response to many forms of cellular stress, to DNA damage (via the caretaker tumor suppressor ATM) and also to oncogene activation (Prives, 1998). When activated, p53 initiates a complex transcriptional response which can induce cell cycle arrest, apoptosis, senescence, DNA repair, angiogenesis or changes in metabolism. One of the p53 targets is *MDM2*, which encodes a nuclear phosphoprotein that inhibits p53's transcriptional activity and targets it for proteasomal degradation, thus creating an important autoregulatory negative feedback loop. MDM2 itself is repressed by the tumor suppressor $p14^{ARF}$, resulting in derepressed p53 function. This adds another instance of p53 regulation.

With regards to cell cycle arrest, *CDKN1A* is one of the most important and best characterized p53 target genes. *CDKN1A* encodes $p21^{CKI}$ - a cyclin-dependent kinase inhibitor with a fundamental role for the regulation of the cell cycle progression at G1. $P21^{CKI}$ binds and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, thereby executing p53-mediated G1 arrest (Luo et al., 1995).

In murine neural stem cells, loss of *Trp53* results in increased neural stem/progenitor cell self-renewal and proliferation *in vitro* and *in vivo* without any effect on differentiation (Meletis et al., 2006). This shows that *Trp53* controls and limits stem/progenitor cell proliferation without affecting cell identity. However, combined knock-out of *Trp53* and *Pten* not only increased self-renewal to a greater extent than in *Trp53* or *Pten* single knock-out cells, but also impaired neural stem cell multilineage differentiation and induced elevated levels of MYC protein (Zheng et al., 2008).

Trp53 null mice display normal embryonal development but are prone to develop different kinds neoplasms by 6 months of age (most frequently: malignant lymphomas and different types of sarcomas; Donehower et al., 1992). In contrast to the *Trp53* null mice, which rarely develop malignant gliomas and rather die from lymphomas or sarcomas, there is an increased incidence of gliomas in patients with the LiFraumeni syndrome, which underscores the importance of *TP53* mutations for gliomagenesis (Malkin et al., 1990).

In human astrocytic gliomas, loss of functional *TP53* is found as an early event, characterizing pathological disease progression of secondary GBMs. Although *TP53* mutations are found less frequent in primary GBMs, the importance of the p53 pathway inactivation for gliomagenesis is

apparent by other genetic alterations, like *MDM2* and *MDM4* amplifications, and deletion/mutations of the gene encoding the MDM2 inhibitor $p14^{ARF}$ (i.e. *CDKN2A*).

3.2.3 CDKN2A tumor suppressor genes

The grave importance of proper function of the p53 response and RB pathway is also underlined by the fact that close regulators of these pathways are common targets of inactivating mutations during tumorigenesis. The INK4 family of CDK inhibitors - including its founding member: $p16^{INK4A}$ (Serrano et al., 1993) - is found inactivated in many tumor types (Ruas and Peters, 1998) including GBMs.

An intriguing surprise was the discovery, that the *P16INK4A* locus (*CDKN2A*) encodes another protein with tumor suppressor function, which has a different exon1 and alternative reading frame for the commonly used exons 2 and 3. This protein is called ARF (alternative reading frame) and is designated as $p14^{ARF}$ in humans and $p19^{ARF}$ in mice (Quelle et al., 1995; Clurman and Groudine, 1998).

INK4A and *ARF* promoters respond to hyperproliferative signals, eg. overactive oncogenic RAS. While ARF represses MDM2, thereby stabilizing p53, INK4A acts by repressing CyclinD/CDK4 thereby preventing the phosphorylation of RB proteins and releasing of E2F transcription factors. Eventually this results in an inhibition of the cell cycle progression (via RB) and also an induction of apoptosis or senescence (via p53). Loss of these tumor suppressors sensitizes cells to transformation by oncogenic RAS (Kamijo et al., 1997) and even single knock-out of *Ink4a* and *Arf* renders mice remarkably tumor-prone (Sharpless et al., 2004). Still, the combined loss of *INK4A* and *ARF* is often observed in many human tumors. In an analysis of genomic aberrations in human primary GBMs the *CDKN2A* locus was found be the most frequently lost genomic region (2008).

3.2.4 BMI1

BMI1 is a polycomb group gene product, that acts as a transcriptional repressor and is a component of the Polycomb Repressor Complex 1 (PRC1), which regulates chromatin changes (Valk-Lingbeek et al., 2004). It was originally identified as a gene cooperating with *Myc* in the generation of B-lymphoid tumors (*Bmi1* = B cell-specific Mo-MLV integration site 1; Haupt et al., 1991; van Lohuizen et al., 1991). Its transformation-supportive role was shown to be mainly due to the repression of the above described tumor suppressors of the *CDKN2A* locus: $p16^{INK4A}$ and $p19^{ARF}$ (Jacobs et al., 1999a; Jacobs et al., 1999b; Lowe and Sherr, 2003). In addition, increased expression levels of *BMI1* were observed in a variety of human epithelial cancers (Song et al., 2010; Voncken et al., 2003; Song et al., 2006; Tateishi et al., 2006; Wang et al., 2008) and brain tumors (Leung et al., 2004; Bruggeman et al., 2007; He et al., 2009), therefore BMI1 is oftentimes referred to as an “oncogene”. Stable knock-down of *BMI1* expression in cultured cancer stem cell-enriched glioblastoma cells impaired their tumorigenic properties *in vitro* and *in vivo*, indicating that steady levels of BMI1 are necessary to counter oncogene-induced tumor suppressor pathways and enabling glioblastoma cells to resist apoptosis (Abdouh et al., 2009)

In the nervous system, BMI1 restricts stem/progenitor self-renewal, as loss of *Bmi1* in mice results in the depletion of the postnatal neural stem cell pool (Molofsky et al., 2003; Park et al., 2003). The impaired neural stem cell self-renewal and proliferation in *Bmi1*-deficient mice was shown to be largely due to derepressed *Ink4a*/*Arf* levels (Molofsky et al., 2003; Bruggeman et al., 2005; Molofsky

et al., 2005). Vice versa: shRNA-mediated knock-down of *Bmi1* resulted in increased *in vitro* self-renewal of fetal and adult neural stem/progenitor cells (Fasano et al., 2007).

BMI1 has a central role in the repression of the tumor suppressors $p16^{INK4A}$ and $p19^{ARF}$ and is therefore situated in a position upstream of all above described tumor suppressor pathways (Fig. 4), exerting a key role for the regulation of apoptosis, and cell cycle progression in normal neural stem/progenitor cells and glioma stem cells. In addition to *INK4A* and *ARF*, other targets of BMI1 with tumor suppressor / cell cycle restricting functions have been identified (e.g. *PTEN* and *CDKN1A*; Fasano et al., 2007).

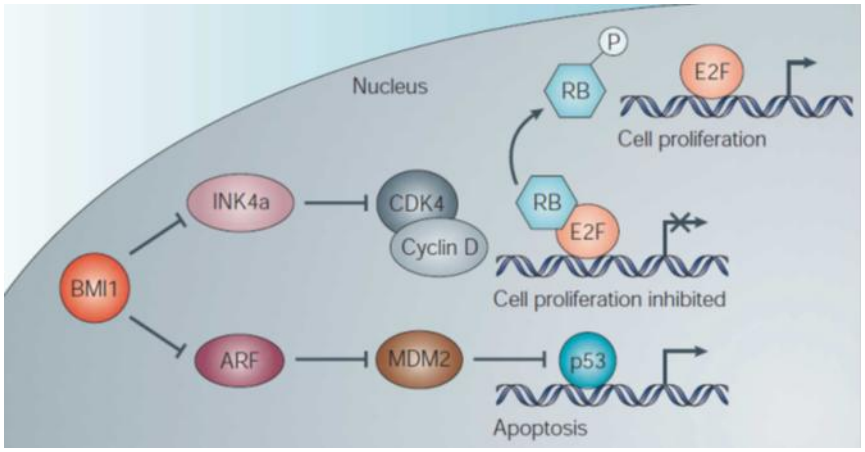

Fig. 4: Tumor suppressor targets of BMI1. Figure taken from Pardal et al. (2003).

Apart from being indispensible for glioma cell maintenance (Abdouh et al., 2009), it still needs to be established that the over-expression of *BMI1* confers tumorigenic properties comparable to increased activity of other glioma oncogenes (*PDGFR*/*EGFR*/*RAS*). The mouse model used by He et al. (2009) showed that *Bmi1* transgene expression under the *Nestin* promoter is not sufficient to initiate brain tumor growth from murine neural progenitor cells.

3.3 The cellular origin of brain tumors

Many experimental models have been applied to identify the cell of origin and pinpoint fundamental molecular hits for the development of different brain tumors. In principle, there are two commonly used modes of experimentation:

1) *Ex vivo* genetic manipulation: This approach includes the specific isolation of a distinct cell type (e.g.: neural stem/progenitor or astrocyte cultures), followed by their oncogenic transformation. The impact on tumorigenic potential is evaluated by subsequent transplantation.

2) *In vivo* targeting: This approach involves the use of transgenic models, in which the candidate cell of origin is targeted by promotor-specific transgene expression/genetic alteration. However, this approach is strictly dependent on established cell lineage-specific promoter activity.

Bachoo et al. (2002) followed the first approach, and isolated and established primary astrocyte cultures from from postnatal day 5 old pups and neurosphere cultures from embryonic day 13.5

brains of *Ink4/Arf* $^{-/-}$ mice. Cells from both, neurosphere and astrocyte cultures were genetically modified *in vitro* to express a variant of mutant activated human *EGFR*. Oncogenic transformation was investigated by orthotopic transplantation of these genetically modified cells into immunocompromised mice. Bachoo et al. (2002) reported that the expression of an oncogenic *EGFR* variant in combination with inactive *Cdkn2a* (*Ink4a*/*Arf*) locus successfully transforms astrocytes and neural stem/progenitor cells into malignant glioma-forming cells. Their results suggest that fetal neural stem cells as well as more differentiated cells from postnatal mice (which would need to acquire long-term self-renewal capacity) might be cells of origin for glioma stem cells.

However, such *ex vivo* approaches might not entirely resemble the physiological situation, as either the identity of the isolated cells is not sufficiently established or as cell culture conditions might enrich for a more immature cell type, which would subsequently be target of the genetic manipulation. Therefore genetic targeting techniques opened better possibilities to prospectively investigate the origin of brain tumors in transgenic mouse models (summarized in Visvader, 2011; Huse and Holland, 2009).

Ding et al. (2001) developed a transgenic mouse model, in which an oncogenic version of human *HRAS* (V12) is expressed under the control of an endogenous astrocyte-specific promoter (*Gfap*). Transgenic animals developed astrocytomas within 3 months after birth. However the exact cellular origin of these tumors could not be established.

Further advanced transgenic models used the RCAS-TVA system (Replication-competent, ALV (avian leucosis virus a)-LTR, splice acceptor). In this system, an oncogene is introduced via the RCAS retrovirus specifically into cells that express the TVA receptor. In transgenic mice, *Tva* can be expressed under the control of a cell type-specific promoter, resulting in cell-type specific targeting of the introduced oncogene. With this method, Holland et al. (2000) could observe that 1) only the combined activation of *Kras* and *Akt* initiates cell transformation resulting in GBM formation in this mouse model, and 2) Nestin-positive progenitors were more susceptible to transformation by *Kras* and *Akt* than *Gfap*-expressing cells. In a similar experimental set up, supratentorial PNETs were generated by *GFAP*-promoter specific expression of human *MYC* and human mutant beta-catenin (*CTNNB1*) in *Trp53*$^{-/-}$ mice (Momota et al., 2008).

Zhu et al. (2005) established a transgenic mouse model including the cre-loxP system to investigate the combined inactivation of *Trp53* and the RAS inhibitor *NF1*. Mice carrying loxP sites flanking the *Nf1* allel were crossed with *Trp53* $^{-/-}$ *hGFAP*-Cre mice. The Cre-recombinase expression was controlled by the human *GFAP* promoter which resulted in *Nf1* deletion in embryonic radial glial cells, mature astrocytes and neurogenic type B astrocytes of the LVW region. Although all GFAP positive mature astrocytes and committed glial progenitors throughout the brain contain the same genetic mutations (*Nf1* and *Trp53* knock-out), as do the neural stem cells in the LVW, Zhu et al. (2005) found that neoplastic transformation is only confined to the LVW region. The authors conclude that either a cell of origin exists in the LVW region, which is expressing the *GFAP* promoter and is more susceptible to *Trp53*/*Nf1* knock-out -mediated astrocytoma formation, or that the microenvironment of the LVW provides a favorable niche for early tumor cells, which either originate from that region or migrate from a more distant region of the brain towards the LVW.

Alcantara-Llaguno et al. (2009) studied the formation of gliomas upon homo- or heterozygous inactivation of *Nf1*, *Pten* and *Trp53* (requiring spontaneous loss of heterozygosity) via the cre-loxP

system. *CreER^{T2}* is expressed under the control of the *Nestin* promoter, however, as the cre-recombinase linked to ER^{T2}, binds to HSP90, it cannot exert its recombinase function in the nucleus until administration of tamoxifen allows for the nuclear translocation of CreER^{T2}. Inactivation of *Nf1*, *Pten* and *Trp53* alleles was initiated by administration of Tamoxifen at embryonic day 13.5 and 4 weeks after birth. Both cases resulted in the formation of high-grade astrocytomas. Using stereotactical adenoviral cre injection, the authors could further demonstrate that cre-mediated tumor suppressor inactivation in non-neurogenic regions does not result in glioma formation, whereas tumor growth is initiated when neural stem/progenitor cells of the LVW are targeted. By visualizing the accumulation of mutant p53 during transformation, Wang et al. (2009) could identify LVW neural stem and progenitor cells as cells of origin of glioma formation.

These observations suggest neural stem cells of the LVW region to be the cells of origin for astrocytic brain tumors, which is supported by a study from Jacques et al. (2009). Similar to the Alcantara Llaguno study, adenoviral-cre mediated inactivation of tumor suppressor genes causes brain tumor initiation in stem/progenitor cells but not in differentiated astrocytes. Interestingly, histologically very different tumor subtypes – dependent on different tumor suppressor gene mutations - developed from common cells of origin. Thus Jacques and colleagues (2009) could demonstrate LVW stem/progenitor cells as cellular origin of gliomas as well as of PNETs.

Persson et al. (2010) investigated the effect of *v-ErbB* (a mutant, activated *EGFR* allele) expression from the *S100ß* promoter. Interestingly, they found that the applied conditions transform oligodendroprecursor cells, resulting in the generation of oligodendrogliomas in the white matter. In contrast in contrast to neural stem cells of the LVW, oligodendroprecursor cells have only a limited self-renewal capacity, and *vErbB* expression is suggested to confer unlimited self-renewal capacity during the transformation of these cells (Persson et al., 2010). Likewise, medulloblastomas developed from neural stem and progenitor cells from the embryonic or early postnatal cerebellum upon genetic interference with the sonic hedgehog pathway (Schuller et al., 2008; Yang et al., 2008).

In summary, there is accumulating evidence that different types of astrocytic brain tumors can develop from embryonic, postnatal and adult neural stem or progenitor cells. Neural stem cells seem to be more prone to oncogenic transformation than more differentiated cells of the postnatal and adult brain such as mature astrocytes (Jacques et al., 2009; Alcantara Llaguno et al., 2009). One explanation for this difference is the fact that neural stem cells already possess a feature which is also a hallmark of tumor cells, namely long-term self-renewal capacity.

ABSTRACTS OF THE MANUSCRIPTS IN THE THESIS

This thesis encompasses the projects listed below. Fulltext of the manuscripts or links to already published papers are given in the Appendix.

Paper 1: Genetic perturbations direct the development of distinct brain tumor types from postnatal neural stem/progenitor cells

Falk Hertwig, Katharina Meyer, Sebastian Braun, Sara Ek, Rainer Spang, Cosima V. Pfenninger, Isabella Artner, Xinbin Chen, Jaclyn A. Biegel, Alexander R. Judkins, Elisabet Englund, Ulrike A. Nuber

Primary brain tumors are classified and treated based on their histological features, however the factors which specify tumor types remain largely unknown. Here we demonstrate that the over-expression of *HRAS* (*V12)* and *MYC* alone or in combination directs the development of glioma, CNS PNET, and atypical teratoid/rhabdoid (AT/RT)-like tumors from neural stem/progenitor cells. Classical AT/RT which lack the tumor suppressor SMARCB1 and AT/RT-like tumors which develop upon the combined over-expression of *HRAS (V12*) and *MYC* are associated with the activation of the unfolded protein response (UPR). We show that malignant rhabdoid tumors with loss of SMARCB1 function display an increased sensitivity toward eIF2alpha phosphorylation – a central UPR component – and propose an interference with the UPR as a novel treatment strategy.

Paper 2: Identification of novel BMI1 targets in neural stem/progenitor cells

Falk Hertwig*, Sebastian Braun*, Ulrike A. Nuber; *These authors contributed equally

Bmi1 was originally identified as a gene that contributes to the development of mouse lymphoma by inhibiting MYC-induced apoptosis via *Ink4a* and *Arf* suppression. BMI1 is a polycomb group protein and acts as a transcriptional repressor by means of chromatin changes. Knock-out and knock-down studies have shown that BMI1 is required for the maintenance of normal postnatal neural stem cells and of brain tumor cells and this function partly relies on the repression of *Ink4a*/*Arf*. Here we show that *Bmi1*-over-expressing postnatal neural stem/progenitor cells display increased self-renewal and survival, however, intracranial transplantations of these cells fail to initiate tumor growth, suggesting that BMI1 rather plays a role as a facilitator of other transforming events. Although the protein is supposed to bind to a large number of genomic regions, the few direct BMI1 target genes described so far likely do not account for the full range of BMI1-mediated neural stem cell effects. We therefore sought to identify novel direct BMI1 targets. Microarray gene expression analysis revealed several genes which are down-regulated in *Bmi1*-over-expressing neurosphere cells, and we show that BMI1 binds to genomic regions of four of them: *Ndn*, *EphA7*, *Trp53bp2*, and *Rps6ka6*. All of these novel BMI1 candidate targets are implied to affect stem cell functions.

Paper 3: CD133 is not present on neurogenic astrocytes in the adult subventricular zone, but on embryonic neural stem cells, ependymal cells, and glioblastoma cells

Cosima V. Pfenninger, Teona Roschupkina, Falk Hertwig, Denise Kottwitz, Elisabet Englund, Johan Bengzon, Sten Eirik Jacobsen, and Ulrike A. Nuber

Human brain tumor stem cells have been enriched using antibodies against the surface protein CD133. An antibody recognizing CD133 also served to isolate normal neural stem cells from fetal human brain, suggesting a possible lineage relationship between normal neural and brain tumor stem cells. Whether CD133-positive brain tumor stem cells can be derived from CD133-positive neural stem or progenitor cells still requires direct experimental evidence, and an important step toward such investigations is the identification and characterization of normal CD133-presenting cells in neurogenic regions of the embryonic and adult brain. Here, we present evidence that CD133 is a marker for embryonic neural stem cells, an intermediate radial glial/ependymal cell type in the early postnatal stage, and for ependymal cells in the adult brain, but not for neurogenic astrocytes in the adult subventricular zone. Our findings suggest two principal possibilities for the origin of brain tumor stem cells: a derivation from *CD133*-expressing cells, which are normally not present in the adult brain (embryonic neural stem cells and an early postnatal intermediate radial glial/ependymal cell type), or from CD133-positive ependymal cells in the adult brain, which are, however, generally regarded as postmitotic. Alternatively, brain tumor stem cells could be derived from proliferative but CD133-negative neurogenic astrocytes in the adult brain. In the latter case, brain tumor development would involve the production of CD133.

Paper 4: Prospectively isolated CD133/CD24-positive ependymal cells from the adult spinal cord and lateral ventricle wall differ in their long-term *in vitro* self-renewal and *in vivo* gene expression

Cosima V. Pfenninger, Christine Steinhoff, Falk Hertwig, and Ulrike A. Nuber

In contrast to ependymal cells located above the subventricular zone (SVZ) of the adult lateral ventricle wall (LVW), adult spinal cord (SC) ependymal cells possess certain neural stem cell characteristics. The molecular basis of this difference is unknown. In this study, antibodies against multiple cell surface markers were applied to isolate pure populations of SC and LVW ependymal cells, which allowed a direct comparison of their *in vitro* behavior and *in vivo* gene expression profile. Isolated $CD133^+/CD24^+/CD45^-/CD34^-$ ependymal cells from the SC displayed *in vitro* self-renewal and differentiation capacity, whereas those from the LVW did not. SC ependymal cells showed a higher expression of several genes involved in cell division, cell cycle regulation, and chromosome stability, which is consistent with a long-term self-renewal capacity, and shared certain transcripts with neural stem cells of the embryonic forebrain. They also expressed several retinoic acid (RA)-regulated genes and responded to RA exposure. LVW ependymal cells showed higher transcript levels of many genes regulated by transforming growth factor-β family members. Among them were *Dlx2*, *Id2*, *Hey1*, which together with *Foxg1* could explain their potential to turn into neuroblasts under certain environmental conditions.

DISCUSSION

1) Identity of normal stem cells in the postnatal central nervous system and their potential to generate brain tumors

During the late 20th century it was still an ongoing debate, whether or not cells of the postnatal mammalian CNS have proliferative potential. Controversial data were published regarding the existence of a neural stem cell in the mammalian brain, which can self-renew and is multipotent, thus has the potential to contribute to regeneration by producing neural cell types (neuron, astrocytes, oligodendrocytes) as progeny. Nowadays it is well established that immature cell types exist in the postnatal mammalian brain, wherein the major neurogenic region, harboring stem cells with long-term self-renewal capability and multipotency is located along the lateral ventricles. The exact identity and location of stem cells at the lateral ventricles has been a matter of debate. Especially the stem cell potential of ependymal cells and the exact location of the type B astrocytic stem cells was discussed controversially (Chojnacki et al., 2009). Only recent papers demonstrated that type B astrocytes are the stem cells in this region, and are located within or close by the ependymal layer (Mirzadeh et al., 2008; Carlen et al., 2009). Therefore in recent publications and this thesis, this neurogenic niche is termed LVW (lateral ventricle wall) region.

1.1 CD133 as a marker for postnatal stem cells in the CNS

CD133 is a transmembrane protein first identified on embryonic and fetal neural stem cells and found to be localized on protrusions like microvilli and cilia (Weigmann et al., 1997; Uchida et al., 2000). In Paper 3 we investigated the presence of CD133 on cell types of the postnatal murine brain, as this protein gained increasing attention in the cancer field. A subpopulation of certain human brain tumor cells (glioma, ependymoma and medulloblastoma) was reported to be specifically enriched with a CD133 antibody, containing the “cancer stem cells” of these tumors. Thus, CD133 became known as a cancer stem cell marker – whether or not it also indicates a link between normal stem cells and CD133-positive brain tumor stem cells, in terms of the cellular origin of brain tumors, remains unclear.

In the LVW region of the postnatal murine brain, we found only two cell types to be CD133-positive: ependymal cells, lining the lateral ventricle wall and a rare subfraction of type B astrocytes, which are interspersed between the ependymal cells and have direct contact to the lateral ventricle. These results were later supported by work from the Alvarez-Buylla group, who showed that a subfraction of single-ciliated type B astrocytes in the ependymal layer (type B1astrocytes) are CD133-positive (Mirzadeh et al., 2008). In our investigation of postnatal LVW cells, we could attribute *in vitro* self-renewal only to CD133 negative cells; however, our data might have been hampered by the isolation techniques we have used. With only a single primary CD133-positive cilium, the CD133 protein might be not abundant enough on the described subfraction of CD133 positive type B1 astrocytes (Mirzadeh et al., 2008), to be isolated by flow cytometry or the epitope is too fragile and might have been lost during the isolation procedure, i.e. these types of stem cells were most likely part of our CD133-negative population. A recent paper from Magdalena Götz’s lab also showed the presence

and *in vivo* self-renewal of a CD133-positive subpopulation of type B1 astrocytes in the LVW (Beckervordersandforth et al.).

The vast majority of CD133-positive cells isolated from the LVW were ependymal cells, which did not display self-renewal capacity. In paper 4 we compared LVW ependymal cells to ependymal cells of the spinal cord which exhibit phenotypic similarities, such as the presence of CD133. In contrast to LVW ependymal cells, we and others showed that CD133-positive ependymal cells from the spinal cord harbor stem cell properties and might have a central role for regeneration in the spinal cord (Paper4; Meletis et al., 2008).

Taken together, our and recently published data indicate the presence of CD133-positive stem cell types in the postnatal CNS: a subfraction of ventricle contacting type B1 astrocytes and spinal cord ependymal cells. In addition, the LVW also contains CD133-negative type B1 neurogenic astrocytes as well as CD133-positive ependymal cells, the latter of which does not possess stem cell properties.

If one considers the CD133 surface marker presence on a postnatal self-renewing cell type sufficient to connect it to a possible origin of brain tumors, these results suggest the CD133-positive subfraction of LVW type B1 cells as well as CD133-positive self-renewing spinal cord ependymal cells as potential cells of origin for CD133-positive brain tumor cells in adulthood (GBMs or SC ependymomas, respectively).

1.2 Activation of self-renewal pathways of tumor cells and postnatal neural stem cells

1.2.1 Ependymoma genes expressed in self-renewing ependymal cells of the spinal cord

Ependymal cell markers, which allow their prospective isolation of ependymal cells by FACS in the postnatal LVW and spinal cord opened the opportunity to directly compare two very similar cell types – both have an ependymal phenotype and function, are multiciliated and CD133-positive – to decipher the molecular factors that underlie one important functional difference: SC ependymal cells, but not LVW ependymal cells are able to self-renew.

We isolated the ependymal cells from both regions using a surface marker combination of $CD133^+/CD24^+/CD45^-/CD34^-$ to exclude other contaminating CD133-positive cells, such as oligodendrocytes, type B cells, hematopoietic and endothelial progenitor cells. We could show that spinal cord ependymal cells are able to self-renew *in vitro* and display multilineage differentiation capacity by generating neuronal, astrocytic and oligodendrocytic progeny in culture. Meletis et al. (2008) showed the self-renewing and multipotent character of this cell type *in vivo*.

In contrast, as shown in Paper 3 and 4, we could not attribute *in vitro* self-renewal potential to LVW ependymal cells, indicating that these cells are post-mitotic under normal conditions. However, Carlen et al. (2009) showed that pathological conditions like stroke, or inhibition of Notch pathway components can be stimulate these cells start to proliferate and generate astrocytic and neuronal progeny. However, they do not self-renew, which leads to a depletion of the ependymal layer.

Among the genes with higher expression in SC compared to LVW ependymal cells, we identified factors which are functionally important for stem and tumor cell self-renewal: *Efnb1*, *Rtel1*, and *Fen1*. EFNB1 participates as a ligand in Ephrin signaling, and has a function in stem/progenitor cell

maintenance by preventing differentiation in the developing cortex (Qiu et al., 2008). *Rtel1* encodes a DNA helicase which regulates telomere length (Uringa et al., 2010; Ding et al., 2004) and RTEL1 variants have been found to be genetic risk factor associated with human malignant glioma cases in the US population (Egan et al., 2011). As an endonuclease, able to resolve stalled replication forks, FEN1 has an important function in processing DNA replication and efficient DNA repair, therefore *Fen1* is required for genomic stability (Shen et al., 2005).

Interestingly, a set of genes specifically up-regulated in SC ependymal cells have been reported as spinal cord ependymoma signature genes: *Hoxb5*, *Hoxc6*, *Hoxa7*, *Hoxb7*, *Vtn*, *Rxrg* (Korshunov et al., 2003; Taylor et al., 2005). In addition, we found a higher expression of *Nf2* in SC compared to LVW ependymal cells. NF2 is a tumor suppressor found inactivated in familial type II neurofibromatosis; its gene product is known to have a central role in restricting cell proliferation in response to contact inhibition (Okada et al. 2007; Curto and McClatchey, 2008). Therefore expression of *Nf2* in SC ependymal cells might represent a key regulatory function, as this tumor suppressor is known to be typically inactivated in spinal cord ependymomas (Ebert et al., 1999).

1.2.2 Self-renewal and anti-apoptotic functions of BMI1 in LVW NSCs

In paper 2 we provide more data contributing to a better understanding of the molecular basis of self-renewal in postnatal LVW stem/progenitor cells. It was previously shown that self-renewal function of neural stem/progenitor cells is dependent on the expression of the polycomb gene and transcriptional repressor *Bmi1* (Molofsky et al., 2005; Fasano et al., 2007). Bmi1 knock-out mice develop normally until birth, but they present tremendous postnatal defects in the hematopoietic and nervous system, which is attributed to an ablation of neural and hematopoietic stem cells. This effect was shown to be mainly caused by BMI1-mediated repression of tumor suppressors $p16^{INK4A}$ and $p19^{ARF}$ (Molofsky et al., 2005).

High expression of *BMI1* in various brain tumors, prompted us to investigate the effect of elevated BMI1 protein levels on neural stem/progenitor cells and potential over-activation of self-renewal pathways. We observed a strong positive effect on *in vitro* self-renewal, inhibition of apoptosis and stimulation of stem cell proliferation upon over-expression of *Bmi1* in neural stem/progenitor cells in culture.

Comparing neural stem/progenitor cells with and without elevated *Bmi1* transcript levels, we identified a variety of genes, directly or indirectly down-regulated by BMI1, with potential anti-apoptotic and tumor repressor functions. As novel direct BMI1 targets we identified: *Ndn*, *EphA7*, *Rps6ka6*, and *Trp53bp2*. *Ndn*, a paternally imprinted gene, is discussed as potential tumor suppressor (Chapman and Knowles, 2009); *EphA7* encodes a signaling receptor involved in regulating apoptosis in neural progenitors (Depaepe et al., 2005). The tumor suppressor gene *Rps6ka6* regulates stress-dependent and replicative senescence (Lopez-Vicente et al., 2009) and *Trp53bp2* is known as an enhancer of pro-apoptotic transactivation functions of p53 (Vives et al., 2006).

In addition, the overall gene expression profile of Bmi1-over-expressing neural stem/progenitor cells indicates that a very immature phenotype is conferred by high *Bmi1* levels: Gene set enrichment analysis (GSEA) revealed that many genes in the *Bmi1* over-expression profile belong to typical embryonic stem (ES) cell expression signatures. As ES cells have an increased risk for tumorigenic

potential due to their immature and proliferative nature, the BMI1-mediated molecular programs might similarly predispose for tumorigenic events. High expression levels of *BMI1* were reported in a variety of human tumors, such as in hematopoietic malignancies (Bea et al., 2001), brain tumors (Bruggeman et al., 2007; He et al., 2009)(Leung et al., 2004), as well as a several epithelial cancers (Song et al., 2006; Tateishi et al., 2006; Wang et al., 2008; Voncken et al., 2003). Furthermore, *Bmi1* was originally identified as a gene cooperating with *Myc* in the generation of B-lymphoid tumors (*Bmi1* = B cell-specific Mo-MLV integration site 1; Haupt et al., 1991; van Lohuizen et al., 1991) and its over-expression has been shown to enable fibroblast immortalization (Jacobs et al., 1999a) and contributes, together with *HRAS*, to the transformation of human mammary epithelial cells (Datta et al., 2007).

In summary, LVW neural stem cells and SC ependymal cells possess self-renewal capacity. Gene expression profiles of SC ependymal cells and SC ependymomas display similarities indicating a functional relationship or even a derivation of adult ependymomas from SC ependymal cells. BMI1 is an important factor for self-renewal of LVW neural stem/progenitor cells; it restricts apoptosis and confers self-renewal. The enforced over-expression of *Bmi1* in these cells results in a variety of transcriptional changes which might facilitate tumor development.

2) Factors leading to brain tumor development

Tumor cells are characterized by aberrant growth, and it is a fundamental question how these cells develop from normal tissue, in which proliferation is tightly regulated. Depending on the cellular origin, the acquisition of self-renewal properties and/or deregulation of proliferation and apoptosis networks is required, involving genetic alterations which activate oncogenes and impair the function of tumor suppressors

2.1 The cellular origin of brain tumors

Irrespective of the uncontrolled growth characteristics, the cellular origin is an important issue. It is crucial to understand tumor development for the prevention of tumorigenesis. However the origin of most brain tumors is unknown and difficult to assess in patients. Two options are possible for the generation of brain tumor cells: the transformation of either neural stem/ progenitor cells, or of differentiated cells like astrocytes. Several lines of evidence strongly support the first option, as 1) Normal neural stem/progenitors cells possess important features crucial for tumorigenesis such as self-renewal 2) Phenotypic and functional markers are shared by normal neural stem cells and tumor stem cells, e.g. the surface protein CD133, or the transcriptional regulator BMI1 (see Papers 2 and 3). Stem cells, which already possess activated self-renewal and proliferation pathways need to acquire additional (epi)genetic events that disable the tight cell cycle control of these cells, resulting in uncontrolled self-renewal. Further differentiated cells, on the other hand, would first have to "reacquire" self-renewal capabilities.

It has been shown in *ex vivo* experiments that cultured astrocytes can be cells of origin for astrocytic brain tumors, when enforcing the expression of a constitutively active variant of the EGF receptor in

cells derived from $p53^{-/-}$ or $Cdkn2a^{-/-}$ (Bachoo et al., 2002; Bruggeman et al., 2007) mice. However the *ex vivo* step might enrich for astrocytic progenitor cells, which have a certain self-renewal potential. A publication by Persson et al. (2010) describes a non-stem cell origin of oligodendrogliomas: Oligodendroprecursor cells, which in contrast to neural stem cells of the LVW have only a limited self-renewal capacity, in part due to the expression of $p27^{KIP}$. A potential mechanism by which these cells might acquire unlimited self-renewal includes v-ErbB-mediated reduction of $p27^{KIP}$ levels (Persson et al., 2010).

Strong lines of evidence show that neural stem progenitor cells serve as origin of brain tumors. Genetically engineered mouse models were used to demonstrate that LVW neural stem cells are the origin of malignant astrocytic tumors (Jacques et al., 2009; Alcantara Llaguno et al., 2009; Wang et al., 2009) as well as for primitive neuroectodermal tumors (Jacques et al., 2009). Self-renewing neural stem/progenitor cells of the embryonal or early post-natal cerebellum have been suggested as origin for medulloblastomas (Schueller et al., 2008, Yang et al., 2008).

Although not proven experimentally, Papers 3/4 in this thesis discuss candidate cells of origin for brain tumors according to the shared expression of the brain tumor stem cell marker CD133. CD133-positive neural stem cell types exist in the developing brain and might be the cellular origin of pediatric tumors, such as supratentorial ependymomas. However, as it is unlikely that these cells persist in the adult brain, other cells such as the CD133-positive subfraction of LVW type B cells are suggested as cells of origin for adult forebrain tumors (see Discussion 1.1).

Using an *ex vivo* approach we tested which genetic perturbations are required to induce tumor development in LVW neural stem/progenitors cells.

2.2 *Trp53* deficiency in combination with *HRAS* or *MYC* are minimal genetic alterations for the generation of brain tumors from LVW neural stem cells

In Paper 1 we focused on the generation of brain tumors from LVW neural stem/progenitor cells, addressing the following question: Which genetic alterations are necessary to transform these cells? The single or combined over-expression of five candidate oncogenes with implied tumorigenic potential in known brain tumors (*MYC*, *HRAS* (*V12*), *Bmi1*, *Ezh2* and *FoxM1*) was tested in cells isolated from WT and *Trp53* knock-out mice. *In vivo* tumorigenic potential was assayed by orthotopic transplantation into syngeneic mice.

Our results showed that the knock-out of the tumor suppressor *Trp53* is required for the oncogenic transformation of postnatal neural stem/progenitor cells - we never observed any tumor formation from genetically engineered wild type (WT) cells. The over-expression of either MYC or HRAS in *Trp53* knock-out cells was already sufficient for tumorigenesis, whereas the over-expression of *FoxM1*, *Ezh2* or Bmi1 alone or in a combination was insufficient for tumorigenic transformation of *Trp53* knock-out cells. Interestingly, three different brain tumors were generated from the same pool of *Trp53* knock-out cells, depending on whether *MYC*, *HRAS* or *MYC* and *RAS* in combination was used (see Discussion below).

Both *HRAS* and *MYC* are obviously potent oncogenes. In normal mitogenic pathways they act as effectors, with resulting stimulation of the cell cycle. Over-activation of these oncogenes renders a

tumor cell independent from outer growth signals which are normally needed to initiate mitogenic pathways and induces constitutively active cell cycle stimulation.

Whereas the expression of *BMI1*/*EZH2* has been reported essential for glioma maintenance (Abdouh et al., 2009), we could not observe an initiation of tumor growth upon over-expression of these genes in WT or *Trp53* knock-out cells. A major part of the BMI1 function relies on its repression of *Ink4a*/*Arf* locus, and equips normal neural cells with a proliferative potential. One could consider *Bmi1* over-expression to have a similar effect as a *Cdkn2a* knock-out. He et al. (2009) showed, that *Bmi1* over-expression does not exhibit an effect on self-renewal in cells which lack *Cdkn2a* expression – i.e. in the developing embryonic brain, and in young postnatal animals. Their *in vivo* data implies that *Bmi1* over-expression in neural progenitor cells does not induce tumors and has only a minor effect on *in vivo* self-renewal (He et al., 2009). Very recently, in contrast to the study from S. Morrison's group (He et al., 2009), S. Marino's group reported a stronger effect of *Bmi1* over-expression on neural stem/progenitor cell self-renewal *in vivo*, but also could not detect tumorigenic properties conferred by elevated BMI1 levels.

Other murine knock-out models imply that gatekeeper tumor suppressor mutations are not the first dominant step during to tumorigenesis: Although neural stem/progenitor cells deficient for *Trp53* or *Pten* have a proliferative phenotype *in vivo*, no development of brain tumors has been described (Meletis et a., 2006; Zheng et al., 2008). However, the combination of several tumor suppressors, eg. *Pten* and *Trp53* (Zheng et al., 2008), *Trp53* and *Nf1* (Zhu et al., 2005; Alcantara Llaguno et al., 2009), *Trp53* and *Rb* (Jacques et al.) lead to glioma formation in from LVW neural stem cells. In addition it is important to note that loss of the RAS-inhibitor NF1 or loss of RB might have stronger effect on proliferation (due to derepressed RAS signaling or released E2F-mediated activation of the cell cycle) than the loss of *Trp53* or *Arf*.

Increased mitogenic signaling might be the prevalent mechanism for glioma formation. EGFR or PDGFR stimulation in postnatal neural stem cells, resulted in pre-neoplastic lesions (Kuhn et al., 1996; Jackson et al., 2006). Also, a mouse model has been established in which expression of an activated form of human *HRAS* (*V12*) as the primary oncogenic event in GFAP positive cells resulted in the formation of astrocytic brain tumors (Ding et al., 2002).

A cellular defense mechanism against this oncogenic signaling is conferred by gatekeeper tumor suppressors, e.g. $p16^{INK4A}$ and $p14^{ARF}$. These tumor suppressors are activated upon aberrant oncogene activation, and can induce apoptosis (via $p14^{ARF}$ – p53 pathway) or cell cycle arrest (via $p16I^{NK4A}$ – RB pathway). However, knock-out of those gatekeepers (like *TP53*, *INKA*, *ARF*) does not directly result in release of E2F from RB resulting in stimulation of cell cycle, hence a further transforming factor is required.

Although p53 has multiple functions which act to protect against tumorigenesis, its counter action against overactive oncogenes (via $p14^{ARF}$ signaling) is discussed as the most important (Serrano 2007). This implies that gatekeeper tumor suppressor functions are crucial after genetic hits which cause oncogenic activation have already occurred. Along these lines of reasoning Sarkisian et al. (2007) describe the stepwise transformation of mammary epithelial cells: Initial low-dose oncogenic activation, which leads to an aberrant mitogenic signaling but is low enough to evade the oncogene-induced senescence response, leads to the secondary acquisition of genetic hits. This can result in spontaneous further up-regulation of the oncogenic pathway, as well as to an oncogene-induced

tumor suppressor response, or it permits the step-wise acquisition of gatekeeper mutations, which results in transformation and leads to the development of breast cancer.

Whether oncogenic activation in general precedes the loss of tumor suppressor function in spontaneous tumors remains unclear. In many tumors that arise from germline mutations, usually the loss of a tumor suppressor allele is followed by a loss of heterozygocity and possible secondary events. This predisposition via a germ-line tumor suppressor mutation is characteristic of several CNS tumors, for example: Neurofibromatosis type I (loss of *NF1*) patients have an increased risk of developing glial tumors, neurofibromatosis type II (loss of *NF2*) patients display an increased risk for schwannomas, or patients with the rhabdoid predisposition syndrome (loss of *SMARCB1*) have an increased risk for malignant rhabdoid tumors.

Fearon and Vogelstein (1990) postulated a sequential acquisition of genetic events starting with activation of oncogenes coupled to the inactivation of tumor suppressors, followed by four to five further mutations before resulting in colorectal carcinogenesis. They also stated that the overall accumulation of events is more important than the order in which they occur – with respect to the biological properties of the tumor.

3) Factors directing tumor phenotype specification

The classification of brain tumors is usually based on histopathological appearance. Although brain tumors are named after their histological resemblance to normal cell types, the cellular origin of most human brain tumor types is unknown. It is also unclear to which extent the cell of origin and/or the genetic changes necessary for transformation specify the resulting tumor phenotype.

3.1 Tumor phenotypes specified by the cells of origin

An elegant model addressing this question has been shown for the transformation of breast epithelial cells: Ince et al. (2007) reported a crucial dependence of the generated tumor phenotype on the cell of origin. After isolation of cells from the same normal breast tissue, different cell culture conditions resulted in the enrichment of two different types of primary epithelial cells. Subsequent transformation with the very same genetic elements led to the development of two different breast tumor types, which exhibited major differences in their histological appearance, as well as in their tumorigenic and metastatic potential.

In the neuroscience field, many subtypes of CNS tumors exist and are classified according to their histopathology (Louis et al., 2007) and resemblance to specific cell types, like ependymoma, oligodendroglioma, astrocytoma, choroid plexus carcinoma. The question whether the cell of origin or the tumorigenic genetic alterations specify the individual tumor phenotypes is difficult to address as cells of origins are unknown for most CNS tumors. For a few brain tumor types, a potential cell of origin has been identified in mouse models, e.g. medulloblastomas arise from cerebellar granule cell precursors (Schuller et al., 2008; Yang et al., 2008), oligodendrogliomas can arise from oligodendroprecursors in the white matter (Persson et al., 2010), astrocytic gliomas can derive from LVW stem/progenitor cells (Alcantara Llaguno et al., 2009).

Interestingly, in one mouse model, Jacques et al. (2009) show that genetic perturbations rather than the cell of origin instruct the tumor phenotype, as different brain tumor types, namely gliomas and PNETs, can be generated from the same cell type, namely postnatal LVW neural stem/progenitor cells.

Experiments in paper 1 of this thesis focused on the generation of brain tumors from the same cell pool, investigating the effect of different genetic perturbations on the tumor phenotype.

3.2 Brain tumor phenotypes specified by genetic changes

LVW stem/ progenitor cells could be established as origin for brain tumors (Jacques et al., 2009; Alcantara Llaguno et al., 2009; Wang et al., 2009). Jacques et al. (2009) did not only succeed in pinpointing the cell of origin, but also showed the impact of different genetic aberrations on the resulting tumor phenotype. The same cell pool could be induced to generate either gliomas or PNETs.

Results from paper 1 of this thesis also show that different genetic events direct the tumor phenotype. These data indicate that three different brain tumor types can develop from the same pool of *Trp53* knock-out neural stem/progenitor cells. The phenotype is dependent on the genetic perturbation: *HRAS* (*V12*) in case of gliomas, *MYC* in the case of PNETs, and a combination of *MYC* and *HRAS* (*V12*) induce an AT/RT-like phenotype. Over-expression of *Bmi1* and *Ezh2* in addition to *MYC* or *HRAS* does not influence the generated tumor phenotype.

Interestingly, RAS signaling – which generates gliomas in our mouse model – is an important feature of gliomas. Although *HRAS* is rarely mutated, elevated *HRAS* levels have been reported in gliomas (Guha et al., 1997) which might arise due to aberrant mitogenic EGFR or PDGRF signaling, due to the loss of the negative regulator *NF1*, or due to copy number alterations of *RAS*/*RAF* genes (Knobbe et al., 2004; Jeuken et al., 2007).

Over-expression of *MYC*, which leads to the development of PNETs in our system, also correlates with the genetics of the human disease, as *MYC* and *MYCN* amplifications are frequently found in as in human CNS PNETs (Behdad and Perry).

The combination of *MYC* and *RAS* generated AT/RT-like tumors in our mouse model. Most cases (70%) of human AT/RTs are characterized by the loss of the tumor suppressor *SMARCB1* (INI1/SNF5/BAF47) (Rorke et al., 1996; Biegel et al., 2002). We could not detect a specific down-regulation or the loss of *SMARCB1* in AT/RT-like tumors compared to gliomas or CNS PNETs. However, we observed that over-expression of MYC and RAS induced a specific gene expression pattern, which includes up-regulation of target genes of the ER-stress pathway (see discussion below). Interestingly, a similar set of genes is expressed in mouse embryonic fibroblasts with *Smarcb1* deletions and the expression of this gene set is also found in the gene expression profile of human rhabdoid tumors (Isakoff et al., 2005).

3.3 Plasticity of generated tumor types

After we observed that the oncogene combination of *MYC* and *HRAS* gave rise to AT/RT-like tumors - a tumor type, which was morphologically completely different from the tumor types generated by single oncogene transductions (gliomas and PNETs) - we addressed whether this phenotype can be generated by sequential genetic alterations.

Fearon and Vogelstein (1990) postulated a sequential acquisition of genetic events in the progression of a tumor type from a low grade to a malignant grade (a sequence from colorectal adenoma to carcinomas), in which they found that the total accumulation of genetic events directs the final malignant properties of the generated tumor, but not the order in which these events occur. This does not entirely relate to the situation of our tumor models, as all three tumors generated are highly malignant tumors. The equivalent human tumors (GBM, CNS PNETs and AT/RT) are classified as WHO grade IV tumors and have a fatal outcome in most patients. When we performed sequential oncogene over-expression we found that *HRAS* over-expression changed the phenotype of (*MYC*-established) PNET-like tumors to AT/RT-like tumors, whereas over-expression of *MYC* in *HRAS*-established gliomas did not change the tumor phenotype.

This indicates that the PNET phenotype generated by *MYC* is not stable and can be changed by the addition of a single gene. This may be due to *MYC*'s function in maintaining an open chromatin state, rendering the cells susceptible to additional (epi-)genetic events. A similar change of an established tumor phenotype (however only *in vitro*) has been reported before; the insertion of *v-HRAS* gene into a small cell lung cancer cell line which harbors amplified *MYC*, leads to the phenotype transition to large cell undifferentiated lung carcinoma *in vitro*. Similar to our system, the transition does not occur if v-HRAS is introduced into small cell lung cancer cells without *MYC* amplifications (Mabry et al., 1988).

Our data and the example of the reported lung cancer phenotype transition implicate that the order of genetic events directs the histopathological appearance as well as the molecular profile of a tumor phenotype. Therefore, in addition to the cell of origin and specific genetic events, an additional parameter is implicated in tumor type specification: The order of genetic events.

4) Tumor cell maintenance

4.1 Maintenance of glioma stem cell self-renewal

In paper 1 we established the formation of three distinct brain tumor types by over-expressing the two oncogenes *HRAS* and *MYC* alone or in combination in *Trp53*-deficient LVW stem/progenitor cells.

In contrast, the over-expression of three other genes investigated in this study (*Bmi1*, *Ezh2* and *FoxM1*) neither conferred tumorigenic potential nor contributed to the specification of the tumor phenotype. As all three have been reported to be expressed at elevated levels in human gliomas (Liu et al., 2006b; Abdouh et al., 2009) it is possible that these genes have an essential function for the maintenance of glioma growth.

BMI1 and EZH2 are polycomb group proteins which act as transcriptional repressors by means of chromatin changes (Sparmann and van Lohuizen, 2006; Bracken and Helin, 2009). *Bmi1* (B cell-

specific Mo-MLV integration site 1) was identified as a gene cooperating with *Myc* in the generation of B-lymphoid tumors (Haupt et al., 1991; van Lohuizen et al., 1991) and the over-expression of *BMI1* enables fibroblast immortalization (Jacobs et al., 1999a) and cooperates with *HRAS* to induce transformation of human mammary epithelial cells (Datta et al., 2007). While in our experiments the over-expression of *Bmi1* and *Ezh2* did not lead to the transformation of *Trp53*-deficient LVW neural stem cells into brain tumors cells, these two polycomb genes have an important role for the maintenance of brain tumors. Both genes are expressed in human glioblastoma cells and were found to be co-expressed with *PROM1* (CD133), indicating a specific function for tumor-initiating self-renewing glioma stem cell population (Abdouh et al., 2009). ShRNA-mediated knock-down of *BMI1* in those cells was shown to deplete the CD133-positive cell population, induce apoptosis via derepression of *INK4a/ARF*, *CDKN1A* and *FOXO3A in vitro* and to diminish *in vivo* tumorigenic potential (as assayed by xenograft transplantations) (Abdouh et al., 2009). Similar to BMI1, it was reported that shRNA-mediated down-regulation of *EZH2* expression in GBM cancer stem cells drastically reduces self-renewal potential *in vitro* and tumorigenic properties *in vivo* (Suva et al., 2009).

For BMI1, an essential maintenance function could be explained by the inhibition of apoptosis (Jacobs et al., 1999a; Abdouh et al., 2009), the maintenance of mitochondrial function and repression of pro-oxidant signaling of p53 (Chatoo et al., 2009; Liu et al., 2009); the latter two are important for glioma cells as they prevent oncogene-induced apoptosis or senescence and to protect from detrimental consequences of oxidative stress. In paper 1 of this thesis, four novel direct BMI1 targets were identified (*Ndn*, *EphA7*, *Rps6ka6*, and *Trp53bp2*). *Ndn* is discussed as potential tumor suppressor (Chapman and Knowles, 2009); *EphA7* encodes a signaling receptor involved in regulating apoptosis in neural progenitors (Depaepe et al., 2005). The tumor suppressor gene *Rps6ka6* regulates stress-dependent and replicative senescence (Lopez-Vicente et al., 2009) and *Trp53bp2* is known as an enhancer of pro-apoptotic transactivation functions of p53 (Vives et al., 2006). Therefore, the transcriptional repression of these genes might be another BMI1-mediated mechanism, which contributes to tumor maintenance.

The forkhead box M1 transcription factor (FOXM1) has a central role for dividing cells. FOXM1 target genes are critically involved in the regulation of various cell cycle checkpoints as well as in the chromosome segregation during mitosis. Therefore FOXM1 is crucial for cell cycle progression and chromosomal stability (Laoukili et al., 2007). *FOXM1* is expressed at elevated levels in a variety of human malignancies as lung cancer, glioblastomas, prostate cancer, basal cell carcinomas, hepatocellular carcinoma, primary breast cancer, and pancreatic cancer (Wang et al., 2010) and references therein). In human primary glioma samples and cell lines, elevated *FOXM1* expression levels have been shown to correlate with increasing malignancy grade (Liu et al., 2006b). While the over-expression of *FOXM1* in low-grade astrocytoma cell lines could confer increased tumorigenic properties, shRNA-mediated knock-down of *FOXM1* expression in a malignant glioma cell line abolished its tumorigenic potential (Liu et al., 2006b). Furthermore, aberrant *FOXM1* levels promote invasive growth and angiogenesis in gliomas (Dai et al., 2007; Zhang et al., 2008).

In our experiments the over-expression of *FoxM1* was not sufficient to induce brain tumor development from *Trp53* knock-out LVW-derived neural stem progenitor cells. However its central role for cell cycle regulation and mitosis renders FOXM1 an essential factor to maintain tumor cell proliferation.

4.2 Coping with cellular stress: the ER stress response pathway in AT/RT-like tumors

As discussed before AT/RT-like tumors could be generated by the combined over-expression of *MYC* and *HRAS* (V12) in LVW-derived *Trp53*-deficient neural stem/progenitor cells (Paper 1). Compared to the other two tumor types generated (gliomas and PNETs), AT/RT-like tumor cells specifically expressed a set of genes associated with the endoplasmic reticulum (ER) stress response. A large number of ER-stress related genes were also found up-regulated in human AT/RTs and in *Smarcb1*-deficient murine embryonic fibroblasts.

The ER stress or unfolded protein response (UPR) encompasses a set of adaptive pathways which serves to cope with different stress conditions, resulting in the accumulation of misfolded proteins, which can arise due to hypoxia or nutrient deprivation in rapidly growing tumors. There are two possible outcomes of this response: the alleviation of the stress condition, resulting in an increased survival, or an induction of cell death in case of intense or persistent ER stress. The outcome is also dependent on the involvement of different sets of regulators of the ER stress response.

In addition to the identified gene expression profile, which implicates an activation of the UPR, histological features like eosinophilic cytoplasmic inclusions were found in some of the generated murine AT/RT-like cells. These inclusions are characteristic for cells with ER stress (Yamagishi et al., 2007), and have also been described as typical features of human AT/RT cells (Biegel et al., 2002).

In murine AT/RT-like cells, the UPR might be induced in response to the over-expression of two dominant oncogenes: *MYC*, which is known to enhance protein translation and oncogenic *HRAS* (*V12*), which has already been reported to induce the UPR pathway (Denoyelle et al., 2006).

Gene expression analysis performed by Isakoff et al. (2005), identified a set of genes with similar expression in human AT/RTs, which are often characterized by the loss of the *SMARCB1*, and in *Smarcb1*-deficient mouse embryonic fibroblasts. Our findings indicate that *SMARCB1* has a functional role in the induction of the UPR. Results obtained in paper 1 suggest that diminished SMARCB1 levels account for an elevated phosphorylation state of eIF2-apha which has a central role in the UPR. Acute eIF2-alpha phosphorylation levels can be pro-apoptotic, whereas sustained eIF2-alpha phosphorylation has been reported to confer survival and protection against stress conditions of rapidly growing tumors, such as hypoxia, oxidative stress, and long-term glucose deprivation (Muaddi et al.; Koumenis et al., 2002; Harding et al., 2003; Bi et al., 2005; Wiseman and Balch, 2005). Our results suggest that reduced or absent *SMARCB1* levels (in the case of AT/RT cells) may change the responsiveness of tumor cells to ER stress via sustained eIF2-alpha phosphorylation. In this way rapidly growing AT/RT cells might cope better with hypoxic and nutrient-deprived conditions. The results in Paper 1 further suggest that this mechanism can be exploited to specifically target *SMARCB1*-deficient tumor cells. The application of the proteasome inhibitor Bortezomib increases the ER stress further and therefore induces apoptosis in cells with reduced or absent *SMARCB1* levels. The potential application of proteasome inhibitors might be of grave therapeutical importance for AT/RTs, as they are difficult to treat with conventional therapies and as no clear treatment guideline have been established for this extremely rare and aggressive childhood tumor (Hilden et al., 2004).

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CURRICULUM VITAE

Falk Hertwig

For reasons of data protection, the CV is not published in the online version of the doctoral thesis.

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, die vorliegende Arbeit selbstständig und ohne unerlaubte Hilfe angefertigt zu haben und alle verwendeten Hilfsmittel und Inhalte aus anderen Quellen als solche kenntlich gemacht zu haben.

Des Weiteren versichere ich, dass die vorliegende Arbeit nie in dieser oder einer anderen Form Gegenstand eines früheren Promotionsverfahrens war.

gez. Falk Hertwig

Berlin, 18.02.2011

APPENDIX (Papers 1 – 4)

Paper 1: Genetic perturbations direct the development of distinct brain tumor types from postnatal neural stem/progenitor cells

Falk Hertwig, Katharina Meyer, Sebastian Braun, Sara Ek, Rainer Spang, Cosima V. Pfenninger, Isabella Artner, Xinbin Chen, Jaclyn A. Biegel, Alexander R. Judkins, Elisabet Englund, Ulrike A. Nuber

Paper 2: Identification of novel BMI1 targets in neural stem/progenitor cells

Falk Hertwig*, Sebastian Braun*, Ulrike A. Nuber; *These authors contributed equally

Paper 3: CD133 is not present on neurogenic astrocytes in the adult subventricular zone, but on embryonic neural stem cells, ependymal cells, and glioblastoma cells

Cosima V. Pfenninger, Teona Roschupkina, Falk Hertwig, Denise Kottwitz, Elisabet Englund, Johan Bengzon, Sten Eirik Jacobsen, and Ulrike A. Nuber

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Paper 4: Prospectively isolated CD133/CD24-positive ependymal cells from the adult spinal cord and lateral ventricle wall differ in their long-term *in vitro* self-renewal and *in vivo* gene expression

Cosima V. Pfenninger, Christine Steinhoff, Falk Hertwig, and Ulrike A. Nuber

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DISCLAIMER:

- Papers 1 and 2 are preprints and produced only for the public examination on this doctoral thesis.

- Paper 1 has been accepted for publication in 'Cancer Research' (*American Association for Cancer Research*) under the title: "Definition of genetic events directing the development of distinct types of brain tumors from postnatal neural stem/progenitor cells" (http://dx.doi.org/10.1158/0008-5472.CAN-11-3525).

- Papers 3 and 4 are not published in the appendix of the online version of this doctoral thesis but can be accessed via their DOI link.

Genetic perturbations direct the development of distinct brain tumor types from postnatal neural stem/progenitor cells

Falk Hertwig[1,2], Katharina Meyer[3], Sebastian Braun[1], Sara Ek[4], Rainer Spang[3], Cosima V. Pfenninger[1], Isabella Artner[1], Xinbin Chen[5], Jaclyn A. Biegel[6], Alexander R. Judkins[6], Elisabet Englund[7], Ulrike A. Nuber[1]

[1] Lund Strategic Research Center for Stem Cell Biology, Dept. of Laboratory Medicine, Lund University, Sweden
[2] Section Biology, Chemistry and Pharmacy, Freie Universität Berlin, Germany
[3] Institute for Functional Genomics, University of Regensburg, Germany
[4] Department of Immunotechnology, Lund University, Sweden
[5] Center for Comparative Oncology, University of California, Davis, USA
[6] Department of Pathology, University of Pennsylvania School of Medicine, The Children's Hospital of Philadelphia, USA
[7] Division of Neuropathology, Department of Pathology and Cytology, Lund University Hospital, Sweden

Correspondence: ulrike.nuber@med.lu.se, phone : ++46 46 222 1563, fax : ++46 46 222 3600

Running title: Genetic perturbations directing brain tumor development

SUMMARY

Primary brain tumors are classified and treated based on their histological features, however the factors which specify tumor types remain largely unknown. Here we demonstrate that the over-expression of *HRAS (V12)* and *MYC* alone or in combination directs the development of glioma, CNS PNET, and atypical teratoid/rhabdoid (AT/RT)-like tumors from neural stem/progenitor cells. Classical AT/RT which lack the tumor suppressor SMARCB1 and AT/RT-like tumors which develop upon the combined over-expression of *HRAS (V12)* and *MYC* are associated with the activation of the unfolded protein response (UPR). We show that malignant rhabdoid tumors with loss of SMARCB1 function display an increased sensitivity toward eIF2alpha phosphorylation – a central UPR component – and propose an interference with the UPR as a novel treatment strategy.

SIGNIFICANCE

This study demonstrates the influence of genes in specifying tumor types and introduces a new concept of tumor type specification: The order of genetic events. Moreover, an involvement of the UPR in AT/RT is shown and this pathway is proposed as a novel therapeutic target for malignancies with loss of the tumor suppressor SMARCB1, such as AT/RT and other malignant rhabdoid tumors.

INTRODUCTION

The pathological classification of brain tumors is based on their histological appearance and treatment strategies primarily rely on this classification. How different cells of origin, cell-intrinsic and extrinsic factors contribute to the development of histologically distinct brain tumor types remains unclear. This issue is central and longstanding in cancer research and has important clinical implications. For example, could histologically very similar tumor types develop from different cells of origin? This possibility is exemplified by medullobastomas and CNS PNETs, both consisting of immature small round cells, and therefore assigned to the group of embryonal tumors. A common cellular origin is rather unlikely since they develop in different brain regions (the cerebellum and the supratentorial brain, respectively). Studies on mouse models have shown that medulloblastomas arise from stem/progenitor cells of the cerebellum (Yang et al., 2008); and (Schuller et al., 2008), whereas CNS PNETs develop from forebrain stem/progenitor cells (Jacques et al., 2010). Alternatively, very different tumor types could develop from common cells of origin which acquired diverse phenotypes due to cell-intrinsic or extrinsic events. In addition to neural stem/progenitor cell origin of murine CNS PNET and medulloblastoma, a number of studies have demonstrated that mouse astrocytomas can arise from embryonic, neonatal, and adult stem/progenitor cells upon different genetic alterations ((Bachoo et al., 2002); (Huse and Holland, 2009) and references therein; (Alcantara Llaguno et al., 2009); (Wang et al., 2009); (Jacques et al.,

2010); (Liu et al., 2010)). Jacques and colleagues provided strong evidence for the potency of single genetic changes and their combinations in directing different brain tumor types from common cells of origin: They described the development of CNS PNET and glioma from postnatal and adult neural stem/progenitor cells depending on different genetic mutations, PTEN/p53 in case of glioma, and Rb/p53 or Rb/p53/PNET in case of CNS PNET (Jacques et al., 2010).

Whether the mere accumulation or the order of single genetic events determines tumor phenotypes and to which extent established tumor types are stable or can be converted to other distinct types by the action of specific genes remains unknown. Here we show that i. Specific genetic perturbations lead to the development of three different brain tumors from postnatal neural stem/progenitor cells, ii. An established *in vivo* tumor type can be converted into another one, and that iii. This is influenced by the order of genetic events. One of the tumor types resembles AT/RT and in a second part of our work we present a so far unrecognized involvement of the UPR in the biology of AT/RT and propose this pathway as a novel therapeutic target for this tumor and related malignancies.

RESULTS

Lack of p53 in combination with the over-expression of multiple genes leads to oncogenic transformation of postnatal neural stem/progenitor cells

In this study, genetic perturbations were conducted to identify genes which lead to the oncogenic transformation of postnatal murine neural stem/progenitor cells. At first, we simultaneously introduced five genes (*Bmi1*, *Ezh2*, FoxM1, V12 mutant of *HRAS* in the following abbreviated as *HRAS*, and *MYC (c-MYC);* altogether designated as "mix") by retroviral infections into wild-type (wt) or p53-deficient neural stem/progenitor cells which were isolated from the lateral ventricle wall (LVW) of postnatal mice and passaged once as neurospheres. The vectors contained the cDNA sequence of one of the five genes followed by an IRES and eGFP gene sequence, allowing us to isolate genetically altered cells by fluorescence activated cell sorting (FACS). Since retroviral vectors only transduce dividing cells, we conclude that dividing neural stem/progenitor cells were targeted in our experiments. After transplantion of these cells into the frontal brain of syngeneic recipient mice, we monitored animals for six months and determined which of the five exogenous genes were expressed in the tumors that had formed. Tumors developed in all three animals that were transplanted with 5,000 or 500,000 mix $p53^{-/-}$ cells, but not in mice that received 500 mix $p53^{-/-}$, or any number of mix wt, empty $p53^{-/-}$, or empty wt cells (Figure 1A). Secondary and tertiary transplantations were performed with FACS-isolated eGFP-positive

tumor cells. RT-PCR using tissue from eight secondary and tertiary tumors revealed expression of exogenous *MYC* and *HRAS* in all investigated tumor samples, *Bmi1* and *Ezh2* in four, and *FoxM1* in three cases (Figure 1B).

HRAS or _MYC_ over-expression in p53$^{-/-}$ postnatal neural stem/progenitor cells generates tumor-initiating cells, whereas _Bmi1_ or _Ezh2_ over-expression alone does not

To test the contribution of the individual genes to the development of brain tumors and to investigate which tumor types develop from postnatal LVW neural stem/progenitor cells, we next conducted a combinatorial gene perturbation study, now only using cells from p53$^{-/-}$ mice and including *MYC*, *HRAS, Bmi1* and *Ezh2*. Each of the four genes was over-expressed alone, five combinations were carried out with two genes over-expressed together and a transduction with empty vector served as control (Figure 2A). To FACS-isolate transduced neurosphere cells expressing two genes, viral vectors expressing *HRAS*-DsRed or *MYC*-DsRed were used in combination with vectors expressing a second gene together with eGFP. After retroviral transductions and FACS-isolation of eGFP-positive neurosphere cells in case of single gene perturbations or eGFP/DsRed-positive cells in case of combined perturbations, 300,000 cells were transplanted into three C57Bl/6 mice, respectively, and animals were monitored for eight months (Figure 2A).

No tumor formation was observed in animals which received the empty vector control cells or cells over-expressing *Bmi1* or *Ezh2* alone (Figure 2A). Tumors

developed in all animals transplanted with cells over-expressing *MYC* alone, or in combination with *HRAS, Bmi1,* or *Ezh2*, as well as in animals that had received *HRAS* and *Bmi1* over-expressing cells (Figure 2A). Tumors were furthermore detected in two out of three animals transplanted with cells transduced with *HRAS alone or HRAS* plus *Ezh2* (Figure 2A).

To assay the *in vivo* self-renewal capacity and stability of tumor types, we performed serial transplantations with FACS-isolated tumor cells (Figure 2A). These experiments revealed a high frequency of cells with tumor-forming capacity among unfractionated tumor cells. To investigate whether the tumor cells continued to express the introduced genes, cryosections were analyzed for eGFP and DsRed fluorescence. Brain tumors which developed from all seven tumorigenic gene combinations were examined, including secondary tumor specimens of all but one gene combination (*HRAS* + *Ezh2*). DsRed and eGFP were detected in all investigated tumor samples, albeit at different levels (see Figures 2B-D and data not shown).

Over-expression of *HRAS* or *MYC* alone or in combination leads to the development of three distinct brain tumor types from p53$^{-/-}$ postnatal neural stem/progenitor cells

Three different tumor types developed according to histological analyses, which included samples from primary, secondary, tertiary, and quaternary transplantations: Primitive neuroectodermal tumors (PNETs), high grade gliomas, and pleomorphic epithelioid tumors with abundant giant rhabdoid cells, which we

refer to as AT/RT-like (Figure 3 and Figure S1). All gliomas developed from cells of three different genetic perturbations: *HRAS* + *Bmi1*, *HRAS* + *Ezh2*, or *HRAS* alone (upper panel in Figure 3 and upper two panels in Figure S1). The PNETs developed from cells over-expressing *MYC* + *Bmi1*, *MYC* + *Ezh2*, and *MYC* alone (middle panel in Figure 3 and lower two panels in Figure S1). The third tumor type among our samples differed from the other two as tumor cells were of varying size with a round to polygonal shape, prominent nucleoli, and several cells had a large eosinophilic cytoplasm and a peripherally located nucleus (lowest panel in Figure 3). Based on these features, we termed these tumors AT/RT-like. Interestingly, all AT/RT-like tumors developed from cells that over-expressed *HRAS and MYC* together – a combination of two oncogenes, which alone served as strong determinants for each of the other tumor types, high grade glioma (*HRAS*) or PNET (*MYC*). The AT/RT-like tumors differed not only histologically from the other two types, but also developed faster (Figure 2E). The general histological features of the three tumor types remained stable throughout serial transplantations, although we want to point out that only secondary transplantations of *HRAS* + *MYC* over-expressing tumors were performed.

The vast majority of tumor cells in the glioma samples produced tenascin C (Figure 4A and left panels Figure S2A and S2B), whereas in CNS PNET and AT/RT-like samples, this protein was present at the tumor borders and blood vessels, but absent in the tumor cells themselves (Figure 4A and left panels Figures S2C and S2D). These results are in accordance with tenascin C localization reported for human brain tumors, as this protein is typically found in

the tumor stroma, and except for high grade glioma, is rarely detected in the tumor cell cytoplasm (Hirata et al., 2009). No GFAP-positive glioma cells were found, and few tumor cells in some of the PNET and AT/RT-like samples showed GFAP staining (Figure 4A and left panels in Figures S2A-D). It should however be noted, that the cells from which these tumors originated, $p53^{-/-}$ postnatal neural stem/progenitor cells, were initially GFAP-positive (Figure 4B) and that reduced or lost GFAP can be seen in high grade gliomas. A widespread vimentin staining was detected in glioma and AT/RT-like tumors, whereas only blood vessels and reactive glia were vimentin-positive in the PNET samples (Figure 4A and left panels in Figures S2A-D). PNET tumor cells, but not glioma or AT/RT-like tumors, produced the neuronal protein ELAVL3/4 (HUC/D) and single cytokeratin-positive cells were only found in AT/RT-like tumors (Figure 4A and left panels in Figures S2A-D). Based on all these findings, we conclude that different genetic perturbations of murine postnatal neural stem/progenitor cells lead to the development of three highly malignant brain tumors, which best correspond to high grade glioma, CNS PNET (considering the forebrain origin of the neural stem/progenitor cells and the forebrain transplantation site), and an AT/RT-like tumor.

Gene expression analyses reveal distinct molecular signatures of the three tumor cell types

We next performed genome-wide expression analyses of tumor spheres that developed under neural stem cell culture conditions from FACS-isolated brain

tumor cells. An unsupervised cluster analysis of the microarray gene expression data from 26 tumor sphere samples (13 CNS PNET, seven glioma, and six AT/RT-like tumors, see Table S1) revealed a clustering into groups corresponding to the histological classification (Figure 5A). Hence, the tumor type induces the dominant differences between the expression profiles. A shrunken centroid classifier (Tibshirani et al., 2002) trained on truncated profiles including the 5000 most variable genes across all 26 samples (Figure 5B) predicted all tumor types correctly in cross validation, thus showing that individual tumors can be reliably classified based on expression profiles of tumor spheres. In fact, only seven signature genes were sufficient for an error-free prediction of tumor types (Figure 5B and 5C). Since the product of one of the seven genes (*Spp1*) has been suggested as diagnostic AT/RT marker (Kao et al., 2005), we tested its expression on the protein level. Immunostainings of tissue sections of the three tumor types confirmed the differential expression of *Spp1* (table in Figure 4A, Figure 5D and left panels in Figures S2A-D). Although seven genes were sufficient for tumor classification, many more genes showed elevated or repressed expression in one of the tumor types relative to the two others (Figure 6A).

Gene set enrichment analyses (GSEA) were performed to investigate if functionally related genes can be assigned to the three tumors. The curated canonical pathways of the Molecular Signatures Database (MSigDB) and manually edited signal transduction pathways, most of which corresponding to the Cancer Cell Map entries, were grouped as pathway gene sets (experimental

procedures and File S1). In addition, gene sets comprising embryonic stem cell (ESC), adult stem cell, MYC-related genes, and marker genes for pediatric human tumors (File S1) were used. The GSEA tool analyzes the gene expression profile of the three tumor types (26 samples in total) in a pair-wise manner; thus the enrichment of specific gene sets is analyzed for two tumor types at a time. These analyses revealed that many gene expression differences between the three tumor types in this study can be explained as downstream *HRAS* or *MYC* effects. A MYC-related gene expression signature that is also part of the ESC profile ((Wong et al., 2008); (Kim et al., 2010)) was enriched in AT/RT-like tumors and CNS PNET (Figure 6B). Furthermore, the functional gene categories related to ribosomes and mRNA processing, which are enriched in the CNS PNET, and the gene sets “aminoacyl-tRNA biosynthesis” and “DNA replication”, which are found in the AT/RT-like type in comparison to glioma (Table S5) belong to known MYC target gene networks (Dang et al., 2006). According to Wong et al. (2008), *MYC* leads to a suppression of the adult tissue stem cell module, which is reflected by the enrichment of this module in the glioma profiles induced by *HRAS* in comparison to the two *MYC* over-expressing tumor types (Figure 6B). Gene sets enriched in the two *HRAS* over-expressing tumors (glioma and AT/RT-like) in comparison to CNS PNET encompassed cytokine/hormone cascades and one set that included genes encoding for ECM and intermediate filament proteins (Table S5). Several of these are known to be regulated by RAS family members.

The *MYC*-induced CNS PNET phenotype is unstable and can be converted into AT/RT-like tumors upon consecutive genetic perturbations

Having shown that tumor types which originate from postnatal neural stem/progenitor cells are specified by the expression of specific oncogenes, we next asked how stable an established tumor phenotype is and whether it can be converted into another type by a consecutive genetic perturbation. Transplantion of 500,000 $p53^{-/-}$ neural stem/progenitor cells over-expressing *HRAS-DsRed* or *MYC-eGFP* generated glioma and CNS PNET tumors. Cells from two different glioma and two different CNS PNET tumors were FACS-isolated based on their red or green fluorescence, respectively. The glioma tumor cells were then transduced with viral vectors over-expressing *MYC*-eGFP, and the CNS PNET cells with vectors over-expressing *HRAS-DsRed*. Double positive cells were sorted and transplanted into recipient mice (triplicates of 500,000 and 5,000 cells in one experiment and triplicates of 5,000 cells in a second experiment). Histological analyses of the mother tumors and the tumors which developed upon consecutive over-expression of *MYC* or *HRAS* revealed that these daughter tumors co-expressed DsRed and eGFP (data not shown). The glioma daughter tumors retained most of their original features - except for the loss of tenascin C in daughter tumors derived from one of the two gliomas (Figures S2A and S2B) - whereas the CNS PNET tumors were converted into AT/RT-like types according to their histology and immunohistochemical characteristics (Figures S2C and S2D). These CNS PNET daughter tumors consisted of cells with a rhabdoid morphology, were positive for markers of the AT/RT-like tumors

(vimentin, cytokeratin and SPP1), and lost the ELAVL3/4 marker which was characteristic for the CNS PNET mother tumors. Microarray gene expression profiles of FACS-purified tumor sphere cells derived from mother tumors (two glioma, one CNS PNET sample), and from six daughter tumors (all samples are listed in Table S1) were determined. Cluster analyses revealed that the glioma daughter tumors which developed upon *MYC* over-expression in *HRAS* induced gliomas fell into the glioma profile group, whereas tumors generated by over-expressing *HRAS* in CNS PNET tumors fell into the AT/RT group (Figure 7A). In summary, our histological and gene expression data not only indicate that certain *in vivo* tumor phenotypes are plastic and can be changed by additional genetic events, but also that the order of genetic events (over-expression of *HRAS* followed by *MYC* or *MYC* followed by *HRAS)* leads to the development of different *in vivo* tumor types (Figure 7B).

Genes related to the unfolded protein response (UPR) are part of the AT/RT-like tumor gene expression profile

Classical AT/RTs are characterized by loss of function of the tumor suppressor SMARCB1 (SMARCB1/SNF5/INI1/BAF47 protein). In our AT/RT-like samples however, no reduced *Smarcb1* RNA levels were found in comparison to the two other tumor types, and SMARCB1 protein was detected in AT/RT-like tumor sections (not shown). To gain a better understanding of the molecular mechanisms leading to the AT/RT-like phenotype, we took a closer look at the genes of group A, which are higher expressed in this tumor in comparison to the

PNET and glioma cells. Whole Genome rVISTA analysis of transcription factor binding sites (Frazer et al., 2004) located in 2500 bp upstream regions of group A genes indicated only one over-represented conserved binding site motif with a p-value <0.005, namely NFE2 (Table S3). The NFE2 sequence motif was originally named after a binding site of the hematopoietic transcription factor NF-E2. However, the motif was later found to be also recognized by five other transcription factors (NRF1-3, BACH1-2), which together form the Cap 'n' collar (CNC) family of transcription regulators (Motohashi and Yamamoto, 2004). We furthermore found that several group A genes are known targets of one CNC protein which binds to NFE2 motifs, NRF2 (=NFE2L2), and of NF-kB (Table S3). Both transcription factors are effector components of a signaling network called the UPR (Healy et al., 2009). This network is activated when the amount of unfolded proteins in the endoplasmic reticulum exceeds its folding capacity (also called endoplasmic reticulum (ER) stress). Known UPR triggers in the context of tumors are for example hypoxia, amino acid and glucose starvation. ER stress pathways can be divided into three branches (PERK-eIF2alpha, ATF6, IRE-XBP1) whose downstream consequences lead to cellular adaptation or death. Altogether, 45 out of 133 genes (33.8%) genes of group A, which are higher expressed in the AT/RT-like tumor were identified as ER stress related (Table S3).

To explore whether an association to ER stress can also be found in human AT/RT tumors and cell culture models of this tumor, we analyzed a list of genes which were previously identified as up-regulated in human AT/RT and also up-

regulated in mouse embryonic fibroblasts upon deletion of the AT/RT tumor suppressor gene *Smarcb1 (Snf5)* (Isakoff et al., 2005). Literature searches revealed that 36 of these 117 genes (30.8%) are related to ER stress (Table S4). This association could not be detected by GSEA, since this pathway was not contained in respective databases. An involvement of the UPR in AT/RT biology has not been reported so far. Using an MCF7 breast cancer cell line, in which *SMARCB1* knockdown can be induced by tetracycline (Xu et al., 2010), we found an increased phosphorylation of eIF2alpha upon SMARCB1 reduction (Fig. 8A). EIF2alpha is a central component of one of the three UPR branches and can be phosphorylated by four different kinases (PERK; GCN2, HRI, PKR) (Fig. 8D). The reverse reaction, dephosphorylation of eIF2alpha, is performed by the catalytic subunit of protein phosphatase-1 (PP1c). SMARCB1 has previously been reported to bind to the catalytic subunit of PP1 (PP1c) and to the PP1 regulatory subunit 15 (PPP1R15A/GADD34), and was shown to increase PP1c activity in solution (Wu et al., 2002). Thus, a likely explanation for increased eIF2alpha phosphorylation in *SMARCB1* knockdown cells is a diminished PP1c activity. Elevated eIF2alpha phosphorylation levels were also detected upon Thapsigargin-mediated ER stress induction in two human SMARCB1-negative tumor cell lines in comparison to three SMARCB1-positive brain tumor cells lines (Fig. 8B). Since phosphorylation of eIF2alpha is known to enhance apoptosis in combination with proteasome inhibition, we tested if the over-activation of the eIF2alpha branch in AT/RT can be therapeutically exploited. Indeed, we found that the treatment of MCF7-SMARCB1 knockdown cells with the proteasome

inhibitor Bortezomib results in an increased number of apoptotic cells as determined by FACS analyses of Annexin V / 7AAD-stained cells (Fig. 8C).

DISCUSSION

Genetic perturbations direct the development of three distinct brain tumor types from the same postnatal neural stem/progenitor cell pool

Our investigations revealed several important and novel aspects concerning the origin of different brain tumors, the role of genetic alterations in specifying tumor phenotypes, and the involvement of the UPR in AT/RT biology.

The origin of most brain tumors is unknown and it remains unclear to which extent the cell of origin and genetic factors determine brain tumor phenotypes. Concerning the cellular origin of the three brain tumor types described here, accumulating evidence indicates that astrocytomas can arise from postnatal and adult neural stem or progenitor cells, and a study by Jacques et al. (2010) showed that both, murine glioma and CNS PNET, can develop from these cells. The origin of AT/RT is unknown. Here we demonstrate that three brain tumor types can develop from the same pool of mouse $p53^{-/-}$ postnatal neural stem/progenitor cells and that their generation is directed by different genetic perturbations: *HRAS* in case of glioma, *MYC* in case of CNS PNET, and *HRAS* + *MYC* in case of the AT/RT-like type. The over-expression of *Bmi1* or *Ezh2* in combination with *MYC* or *HRAS* did not affect the basic histological and molecular features of the CNS PNET or glioma tumors. Our results, together with

the work published by Jacques and colleagues demonstrate that genetic factors play an instructive role in establishing brain tumor phenotypes.

The genes which directed the development of these three murine tumors are also implicated in the pathogenesis of respective human tumor types. P53 is often inactivated in human high grade gliomas (Cancer Genome Atlas Research Network (2008)) and a positive p53 staining is found in CNS PNET and AT/RT indicating a stabilized p53 protein of altered functionality or an altered p53 pathway (Eberhart et al., 2005). *HRAS* mutations are not common in human gliomas, but increased copy numbers of *RAS/RAF* genes and an increased RAS activity has been reported ((Jeuken et al., 2007) (Cancer Genome Atlas Research Network, (2008)). Over-expression of *MYC* led to the development of CNS PNET in our study, and *MYC* and *MYCN* amplifications are a frequent feature of this tumor type (Behdad and Perry, 2010).

Our combined over-expression of *MYC* and *HRAS* in $p53^{-/-}$ postnatal neural stem/progenitor cells resulted in an AT/RT-like tumor. Classical human AT/RT is a very aggressive central nervous system tumor which mainly occurs in infants and children. In the majority of cases, an inactivation of the tumor suppressor gene *SMARCB1 (INI1/SN5)* is found (Biegel et al., 1999). Although no loss of function of this gene was detected in our murine tumors, we found that the gene expression pattern of our AT/RT-like tumors shares striking similarities with human AT/RT and with mouse fibroblasts lacking *Smarcb1* (see discussion below).

The GSEA provided molecular support for our histological classification of the three generated murine tumor types. Two findings of this analysis however need to be discussed. Our mouse CNS PNET profile was most similar to the human medulloblastoma gene set, whereas the human PNET gene set was not enriched. Two reasons likely account for this result: i. The human PNET gene set does not provide a representative signature of this tumor. ii. Medulloblastomas and CNS PNETs are more similar to each other than to glioma and AT/RT. In particular, the human medulloblastoma gene set included the *MYCN* gene and many *MYC* target genes, which explains the similarity to our *MYC*-induced CNS PNET profile. The second result that requires discussion concerns the enrichment of the human AT/RT gene set in both our murine AT/RT-like and glioma tumors in comparison to the murine CNS PNET. Apparently, the human AT/RT gene set contains genes which are also found in the mouse glioma profile and are significantly less enriched in the mouse PNET profile, as this assignment only occurred in this pair-wise comparison.

Strong *HRAS*, *MYC* and *HRAS+MYC* effects on the gene expression profile of tumorigenic cells

Genome-wide gene expression analyses revealed that the histological grouping into glioma, CNS PNET, and AT/RT-like tumors also corresponds to distinct molecular clusters, confirming the strong gene expression effect of the genetic perturbations. The GSEA showed that many gene expression differences between the three tumors are *MYC* or *HRAS* effects. A classification of the three

tumor entities was already possible based on the expression information of seven genes, which indicates their clear-cut molecular difference. One of these genes was *Spp1*, which codes for secreted phosphoprotein I (osteopontin), and is highly expressed in the glioma and AT/RT-like tumor, but not in the CNS PNET. This expression pattern was also confirmed on the protein level by staining tumor tissue sections. SPP1 protein levels have been reported to correlate with the histological grade of astrocytomas (Toy et al., 2009) and to be significantly higher in AT/RT than in medulloblastoma tissue (Kao et al., 2005).

A genetic alteration can shift the phenotype of an established tumor type to another one and the order of genetic events can specify tumor phenotypes

Our experimental system not only allowed us to study the outcome of multiple simultaneous genetic alterations, but also the effect of sequential ones. Thus, we could address two other fundamental questions, namely i. If and to what extent genetic alterations can shift established *in vivo* tumor phenotypes and ii. Whether the order of genetic alterations or the mere accumulation of specific changes specifies a tumor phenotype. In addition to well-known phenotypic and genetic changes that occur during the progression of the same tumor type (from lower to higher grade tumors, or benign to malignant lesions, such as the adenoma-carcinoma sequence; Fearon and Vogelstein, Cell, 1990), the co-existence or sequential appearance of two distinct malignant tumor phenotypes in the same tissue has been described. Our findings indicate that an established *in vivo* tumor type (CNS PNET) can shift to another one (AT/RT-like tumor) (Figure 7B) and

that this process can be driven by the over-expression of a single gene (*HRAS*). Several lines of evidence argue against the development of this second tumor type from a pre-cancerous progenitor cell or another brain cell type that might have been present: We FACS-isolated tumor cells from the mother tumor based on eGFP, which was co-expressed with *MYC* in the $p53^{-/-}$ neural stem/progenitor cells giving rise to the initial tumor, thus the cells used for the secondary genetic perturbation were $p53^{-/-}$ *MYC* expressing cells. The tumors which developed consisted of cells double positive for eGFP and DsRed, the latter being co-expressed together with the subsequently introduced *HRAS* gene. Moreover, the high frequency of CNS PNET development observed in the first part of our study upon transplantation of low numbers of $p53^{-/-}$ *MYC* cells – even under conditions which could be further improved – indicates that potentially every tumor cell from such *MYC*-initiated tumors is tumorigenic. Mabry and colleagues have shown earlier that the insertion of the v-Ha-*ras* gene in a small cell lung cancer cell line that carries an amplified *MYC* gene induces a transition to a large cell undifferentiated lung carcinoma *in vitro*, whereas no transition occurs when v-Ha-*ras* is inserted in small cell lung cancer cell lines without *MYC* amplification (Mabry et al., 1988). Here we report that a single gene can trigger the transitions of an *in vivo* tumor type.

Our results demonstrate that the sequence of genetic changes can specify *in vivo* tumor types, which differ in their histological appearance, molecular profile, and growth rate: *MYC* followed by *HRAS* over-expression leads to AT/RT-like tumors, whereas the reverse order results in the persistence of the glioma

phenotype. Previous reports have shown that the order of transcription factor gene expression directs the specification of normal hematopoietic cells (Iwasaki et al., 2006) and that the relative order of *K-ras* and *APC* gene mutations determines the grade, but not the tumor type in case of lung neoplasia (Johnson et al., 2001). With the reservation that our system is based on a strong expression of *MYC* and *HRAS* under the CMV promoter and includes an *ex-vivo* step, we provide proof-of-principle that the order of genetic events can specify *in vivo* tumor types. It is conceivable that MYC over-expressing tumors are particularly plastic and prone to phenotype transitions as MYC promotes cellular reprogramming into induced puripotent stem cells which possess a broad developmental potential (Nakagawa et al., 2010). A possible explanation for the failure of *MYC* to turn *HRAS* over-expressing glioma cells into AT/RT-like tumors is the inaccessibility of certain binding sites for this transcription factor in the glioma cells, which would be required to regulate genes determining the AT/RT-like type. Taken together, we propose an additional parameter to influence the specification of tumor types: Cell-of-origin, genetic and epigenetic events, and the order of genetic events.

AT/RT and AT/RT-like tumors are characterized by an activated UPR

Our results suggest a particular involvement of the UPR in AT/RT biology. This signaling network serves as a cellular mechanism to cope with stress conditions that lead to increased protein misfolding, such as hypoxia and nutrient deprivation in rapidly growing tumors. Activation of the UPR can have different

consequences - increased survival or cell death. These outcomes seem to depend on the involvement of the three different UPR branches and on the dynamics of the response.

Of our three generated murine brain tumors, the one with the highest growth rate – the AT/RT-like tumor – shows a particular activation of the UPR according to its gene expression profile. Moreover, a frequent feature of AT/RT, eosinophilic cytoplasmic inclusions, are found in cells under ER stress (Yamagishi et al., 2007) and in some of our AT/RT-like tumors. An activated UPR could result from reduced glucose availability due to the rapid growth of these tumor spheres. Another explanation for the activation of this pathway could be related to specific contributions of *MYC* and *RAS*: MYC is known to enhance protein translation, thus increasing the protein load in the ER, and the mutated form of *HRAS* used in our study (*V12 HRAS*) has been shown to particularly induce the UPR pathway (Denoyelle et al., 2006).

With regards to classical human AT/RTs which lack the tumor suppressor gene *SMARCB1* and *Smarcb1*-deficient mouse embryonic fibroblasts, we found many UPR-related genes in their published gene expression signatures. Most notably, our experimental results suggest that reduced levels of SMARCB1 protein, which has previously been shown to activate PP1c-GADD34 in solution (Wu et al., 2002), accounts for an elevated sensitivity toward eIF2alpha phosphorylation, a central UPR mechanism (Figure 8D). In contrast to acute eIF2alpha phosphorylation, which can be pro-apoptotic, sustained eIF2alpha phosphorylation has been shown to confer cytoprotection against hypoxia,

oxidative stress, and long term glucose deficiency, all of which representing typical *in vivo* conditions of rapidly growing tumors ((Koumenis et al., 2002); (Harding et al., 2003); (Bi et al., 2005); (Wiseman and Balch, 2005); (Muaddi et al., 2010)). Here we show that reduced or absent levels of the tumor suppressor *SMARCB1* result in a cellular state which is characterized by an elevated sensitivity toward eIF2alpha phosphorylation. Importantly, we demonstrate that the involvement of SMARCB1 in regulating the UPR could be therapeutically exploited: Application of the proteasome inhibitor Bortezomib, which is known to increase ER stress and to synergize with a chemical inhibitor of the GADD34-PP1c complex (Schewe and Aguirre-Ghiso, 2009), leads to apoptosis of cells with reduced *SMARCB1* levels. Such an approach could represent a novel strategy for treating AT/RT and related malignancies with *SMARCB1* loss-of-function.

EXPERIMENTAL PROCEDURES

Detailed experimental information is given in the supplemental experimental procedures.

Animal procedures

C57Bl/6J and p53 knockout mice (TSG-p53) were from Taconic Europe (Denmark). All animal procedures were performed with consent from the ethical

committee at Lund University. Transplantations into the right frontal brain lobe of 4-8 week-old C57Bl/6J mice were carried out.

Neurosphere and tumorsphere culture

LVW tissue from 4 week-old mice and brain tumor tissue was dissected, digested with Accutase (PAA, Austria), and filtrated. Brain tumor cells were FACS-isolated based on eGFP and DsRed expression. Cells were cultured as spheres in DMEM/F12 (1:1) with Glutamax, B27, penicillin (100 units/mL), streptomycin (100 µg/mL; all from Invitrogen), HEPES (10 mmol/L), Partricin (0.5 µg/mL, Biochrom); insulin (20 µg/mL; Sigma Aldrich), recombinant human epidermal growth factor (EGF; 20 ng/mL), and rhFGFbasic (20 ng/mL; PAN Biotech). Depending on the sphere size, cells were passaged 5-7 days after plating.

Viral transduction of neurosphere cells

The pCMMP-IRES2-eGFP retroviral vector was provided by Laurent Roybon (Lund University, Sweden). A vector containing DsRed was generated by replacing the IRES-eGFP sequence with IRES-DsRedExpress. cDNA sequences of human *MYC*, human *V12HRAS*, mouse *Bmi1*, mouse *Ezh2*, and mouse *FoxM1* were inserted upstream of the IRES sequences. The ecotropic retroviral packaging cell line EcoPack2-293 (Clontech) was used to produce viral supernatant.

FACS

Tissue or transduced cells were dissociated as described above, washed twice in PBS/1%BSA, and 7-AAD (Sigma/Merck) was added for dead cell discrimination. Sorting was performed with a FACSVantage system (DiVa option, BD Biosciences), excluding doublets and dead cells. Sorted cells were harvested by centrifugation (5min 200 *g*) and resuspended in fresh growth medium. Cells were cultured at 37°C/5%CO_2 overnight or longer prior to transplantations.

RT-PCR

RNA was isolated (AllPrep DNA/RNA Mini Kit, QIAGEN, Hilden, Germany) and 2 µg of total RNA was used for cDNA synthesis.

Staining of tissue sections

Tissue was fixed in 4% formaldehyde/PBS overnight and subsequently either dehydrated and embedded in paraffin or cryo-protected in 25% sucrose/PBS and embedded in TissueTek OCT compound (Sakura) for crysections. Immunohistochemistry was performed according to the supplemental experimental procedures.

Microarray hybridization and analyses

Isolated total RNA was analyzed on the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Generation of probes, hybridization to Affymetrix Gene 1.0 ST Arrays, washing, and scanning were performed

according to the Affymetrix standard protocol (GeneChip Whole Transcript (WT) Sense Target Labeling Assay User manual, P/N 701880 Rev.5). 300 ng RNA was used for the first strand cDNA synthesis. Microarray data were normalized by the Robust Multi-array Average (RMA) method using the Expression Console software. The data are deposited at http://www.ncbi.nlm.nih.gov/geo/, accession number XXX.

Unsupervised clustering and classification

Genes representing the different tumor types were found by correlating the 5000 most variable genes across all samples to tumor type labels. To see whether the 1000 highest ranked genes were differentially expressed between tumor types a linear model was fitted using the R package limma. The model included the different histological tumor types. All 1000 genes showed a p-value smaller than 10^{-6} and were considered for further analysis.

Complete linkage hierarchical clustering of tumor sphere samples was performed based on euclidean distances calculated between the samples. For supervised classification we used shrunken centroid classification as implemented in the R package PAMr.

GSEA

GSEA was performed based on (Subramanian et al., 2005). The difference of the normalized intensities (i.e. logarithmic values) served as a metric for ranking.

Gene set permutations (1,000) were chosen. Applied gene sets are listed in File S1.

Western blot analysis

Western Blots were performed as described in supplemental experimental procedures. Antibodies used were: anti-BAF47 (SMARCB1/SNF5; BD Biosciences, San Diego, CA), anti-phospho-eIF2alpha (119A11; Cell Signaling), anti-beta actin-HRP (Abcam, Cambridge, UK).

Human cell lines

CHLA-02-ATRT (ATCC) cells, LM malignant rhabdoid tumor cells (Versteege et al., 1998), DAOY medulloblastoma, SW1783 and Hs683 glioma, and MCF7 cells were kept according to the supplemental experimental procedures.

SUPPLEMENTAL DATA

Figures S1 and S2, File S1, Tables S1-S5, and supplemental experimental procedures can be found online.

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FIGURE LEGENDS

Figure 1

Serial intracranial transplantations and expression analyses of genetically perturbed wt and p53$^{-/-}$ neural stem/progenitor cells

(A) Primary transplantations (left hand side) of wt and p53$^{-/-}$ neural stem/progenitor cells transduced either with virus containing an empty vector or with a mixture (Mix) of five different viral supernatants each containing one gene (*Bmi1*, *Ezh2*, *HRAS*, *MYC*, and *FoxM1).* The number of mice which developed tumors/number of transplanted mice is indicated. Secondary transplantations (middle) and tertiary transplantations (right) were performed with FACS-isolated eGFP-positive cells from one of the underlined tumors. Cells from two tumors arising upon primary transplantation of 500,000 Mix p53$^{-/-}$ cells and from one tumor which developed upon transplantation of 5,000 Mix p53$^{-/-}$ cells were used in secondary transplantations (four different cell number categories from less than 12 to 100,000 cells in one case, 100,000 cells in the second, and three different cell number categories in the third case). Tertiary transplantations were performed with cells from two 50 cell secondary tumors).

(B) Agarose gel electrophoresis of RT-PCR products to analyze the expression of the five introduced genes *Bmi1*, *Ezh2*, *HRAS*, *MYC*, and *FoxM1* in developed tumors. Mix $p53^{-/-}$ cells before transplantation were used as positive control and $p53^{-/-}$ cells as negative control. Forward PCR primers are gene-specific and reverse PCR primers bind to a viral vector sequence which is part of the bicistronic transcript.

Figure 2

Serial intracranial transplantations of $p53^{-/-}$ neural stem/progenitor cells expressing different genes (*MYC, HRAS, Bmi1* and *Ezh2*), and of control cells expressing eGFP only

(A) Primary to quaternary transplantations are depicted in four boxes from left to right. The number of mice with tumor development/number of transplanted mice is shown within the boxes. Cells from underlined tumors were FACS-isolated and used in subsequent transplantations. For example, three out of three mice transplanted with 300,000 *MYC* over-expressing cells developed tumors. 50 FACS-isolated cells from each of the three tumors were transplanted into two recipient mice and 5,000 cells from each tumor into one animal. Both animals that had received 50 cells from the third primary tumor developed tumors, and FACS-isolated cells from one of these 50 cell tumors (underlined) were further transplanted into tertiary recipients: one animal received 50 cells and another one 100 cells. Both animals developed a brain tumor, and cells from the first one (underlined) were used in quarternary transplantations (three mice receiving ≤10

cells and one animal receiving 50 cells). In all cases - except for the one in which tumors are marked with a and b - only one tumor sample of each genetic type was used for subsequent transplantations.

Cryostat sections of secondary tumors derived from cells over-expressing *MYC* (B), *HRAS* (C) and *MYC* together with *HRAS* (D) showing green fluorescent (B, C) or green and red fluorescent tumor cells (D). Asterisk: adjacent normal tissue. Scale bars: 50 µm.

(E) Life span of tumors which arose upon primary to quaternary transplantations of 300,000, 5,000, or 50 cells. Tumors are grouped according to their histology: gliomas (over-expressing *HRAS* alone and together with *Bmi1* or *Ezh2,* red dots), CNS PNET (over-expressing *MYC* alone and together with *Bmi1* or *Ezh2,* green squares), AT/RT-like tumors over-expressing *HRAS* together with *MYC (*blue triangles*).* Horizontal lines indicate median lifespan values of animals that were sacrificed due to tumor development (median values from left to right: 35, 31, 8/ 22, 23, 11/ 25, 49, 17). Animals that were alive after eight months (244 days) were not included in the calculation of median values and are indicated on top.

Figure 3

Representative macroscopic and microscopic images of brain tumors which developed upon transplantation of p53$^{-/-}$ neural stem/progenitor cells over-expressing *HRAS*, *MYC*, and *HRAS* together with *MYC*

Hematoxylin/eosin-stained paraffin sections. *HRAS* over-expressing tumors show glioma features with abundant spindle-formed cells, necroses (arrow), and single

giant cells containing pleomorphic and/or multiple nuclei (black arrow heads). Tumors with PNET features developed upon *MYC* over-expression. They consist of small round cells with a large nucleus to cytoplasm ratio, sometimes forming rosette-like structure (magnified section). Tumors derived from neural stem/progenitor cells over-expressing *MYC* together with *HRAS* show AT/RT features. They consist of a large number of rhabdoid cells with eccentric nuclei and prominent nucleoli. Many cells have eosinophilic cytoplasmic inclusions (white arrow head). In addition, smaller cells with scant cytoplasm displaying PNET features are present. Scale bars: 50 µm.

Figure 4

(A) Representative immunohistochemistry images of tumors and (B) GFAP-staining (red) of p53-deficient neurosphere cells derived from the postnatal mouse LVW. Blue staining in (B): DAPI-positive nuclei. Paraffin-embedded tumor sections stained with antibodies against tenascin C (TNC), vimentin (VIM), GFAP, Cytokeratin (CK), and ELAVL3/4 are shown. Protein staining: brown, counterstaining: blue. The table summarizes all immunohistochemistry results. + staining of the majority of tumor cells, (+) weak staining, [+] staining of a few tumor cells. N: adjacent normal brain. In CNS PNET, the vimentin antibody stains blood vessels and reactive glia, and the tenascin C antibody shows a blood vessel-associated staining. Scale bars: 50 µm.

Figure 5

Gene expression analyses of brain tumor cells

(A) Unsupervised clustering of FACS-isolated, sphere-cultured tumor cells from 26 tumors which developed after primary, secondary, and tertiary transplantations based on microarray data. Y-axis: euclidean distance. Three major clusters are identified based on the 5,000 most variable genes. They correspond to the three histological tumor types. Internal array sample numbers are shown (see also Table S1).

(B) Prediction analysis for microarrays using the 5,000 most variable genes over all 26 samples. Different threshold values corresponding to different numbers of genes are shown along the x-axis. A classification into the three tumor types can already be done - with minimal cross validation error and considering the risk of overfitting - based on the expression information of a minimum of seven genes.

(C) Relative expression values of the seven genes which allow a classification of the three tumor types (y-axis) plotted against the 26 tumor sphere samples (x-axis). Red circles (left side): glioma samples, green circles (middle section): CNS PNET samples, blue circles (right side): AT/RT-like samples.

(D) Confirmation of the differential expression of *Spp1* by immunohistochemistry of paraffin sections. In CNS PNET tumors, SPP1 staining is confined to blood vessels and immune cells, and virtually absent in tumor cells. Scale bars: 50 µm.

Figure 6

Gene groups and gene sets representing the three different tumor types

(A) Color-coded relative gene expression differences of the 1,000 genes with maximum expression correlation to the three tumor types. Genes are plotted along the y-axis and tumor types along the x-axis. Higher expression levels: yellow, lower expression levels: blue. Six groups of genes were identified (A-F) with the highest correlation to one of the three tumor types. Group A is characterized by many genes with NFE2 binding sites in their promoter region, a high number of NFkB and NFE2L2 target genes, and several epithelial cell-expressed genes (see also Table S3).

(B) GSEA to identify molecular signatures of human brain tumors and of mouse stem cells that correlate best to the gene expression profile of the three tumor types. Gene sets with a FWER p-value ≤ 0.01 are shown. Detailed gene set information is given in File S1. a (Wong et al., 2008); b (Kim et al., 2010), c (Pomeroy et al., 2002). NES = absolute normalized enrichment score. Vs=versus.

Figure 7

Microarray data cluster analysis and summary of consecutive perturbation experiments

(A) Unsupervised clustering of the microarray profiles of FACS-isolated tumor sphere cells from the 26 original tumors (see also Fig. 5) together with one CNS PNET and two glioma mother tumors and respective daughter tumors which

developed upon consecutive perturbations. The 1,000 most variable genes were used for clustering. Y-axis shows the euclidean distance. Internal array sample numbers are shown (see also Table S1).

(B) Schematic summary of the consecutive perturbation experiments.

Figure 8

Involvement of SMARCB1 in the UPR

(A) shRNA-mediated SMARCB1 knockdown in MCF7 cells (induced by tetracycline) leads to increased levels of eIF2alpha phosphorylation as determined by immunoblots. ER stress was induced by DTT and thapsigargin (TG) treatment for 3h. Unconventional splicing of XBP1 is shown by RT-PCR as an indicator of another activated UPR branch. XBP1s, XBP1u, XBP1h: spliced, unspliced and hybrid XBP1 cDNA (the latter representing XBP1s/XBP1u heterodimers).

(B) Increased eIF2alpha phosphorylation levels upon three hour TG exposure are detected in the human CHLA2 AT/RT and malignant rhabdoid tumor (MRT) cell line LM, both of which lacking SMARCB1 in comparison to three SMARCB1-positive brain tumor lines (DAOY, SW1783, Hs683). Fold-difference of β-actin-normalized phosphorylated eIF2alpha signal intensities between TSG-treated and untreated cells: AT/RT: 5.89; MRT: 1.27; DAOY: 0.77; SW1783: 0.75; Hs683: 0.81.

(C) Treatment of the MCF7 cell line with the proteasome inhibitor Bortezomib (BTZ) for 12 hours leads to increased apoptosis in tetracycline (Tet)-induced

SMARCB1 knockdown (kd) cells. % Annexin V-positive and 7AAD-negative apoptotic cells of three independent experiments are shown. Error bars: standard error of the mean. P-values of unpaired t-tests comparing non-induced to Tet-induced cells.

(D) Schematic summary showing the connection between SMARCB1 and eIF2alpha phosphorylation.

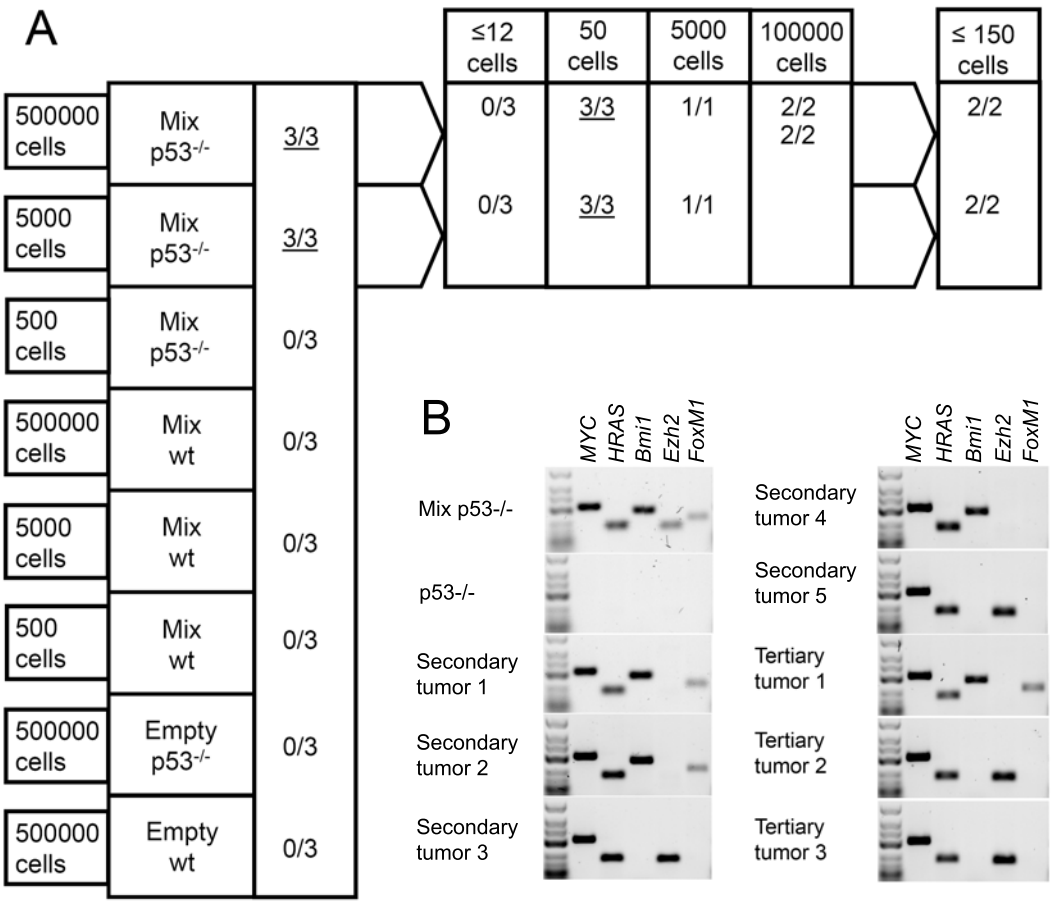

A
500000 cells
5000 cells
500 cells
500000 cells
5000 cells
500 cells
500000 cells
500000 cells
Mix p53-/-
Mix p53-/-
Mix p53-/-
Mix wt
Mix wt
Mix wt
Empty p53-/-
Empty wt
3/3
3/3
0/3
0/3
0/3
0/3
0/3
0/3
≤12 cells
50 cells
5000 cells
100000 cells
≤ 150 cells
0/3
0/3
3/3
3/3
1/1
1/1
2/2
2/2
2/2
2/2
B
MYC
HRAS
Bmi1
Ezh2
FoxM1
Mix p53-/-
p53-/-
Secondary tumor 1
Secondary tumor 2
Secondary tumor 3
Secondary tumor 4
Secondary tumor 5
Tertiary tumor 1
Tertiary tumor 2
Tertiary tumor 3

FIGURE 1

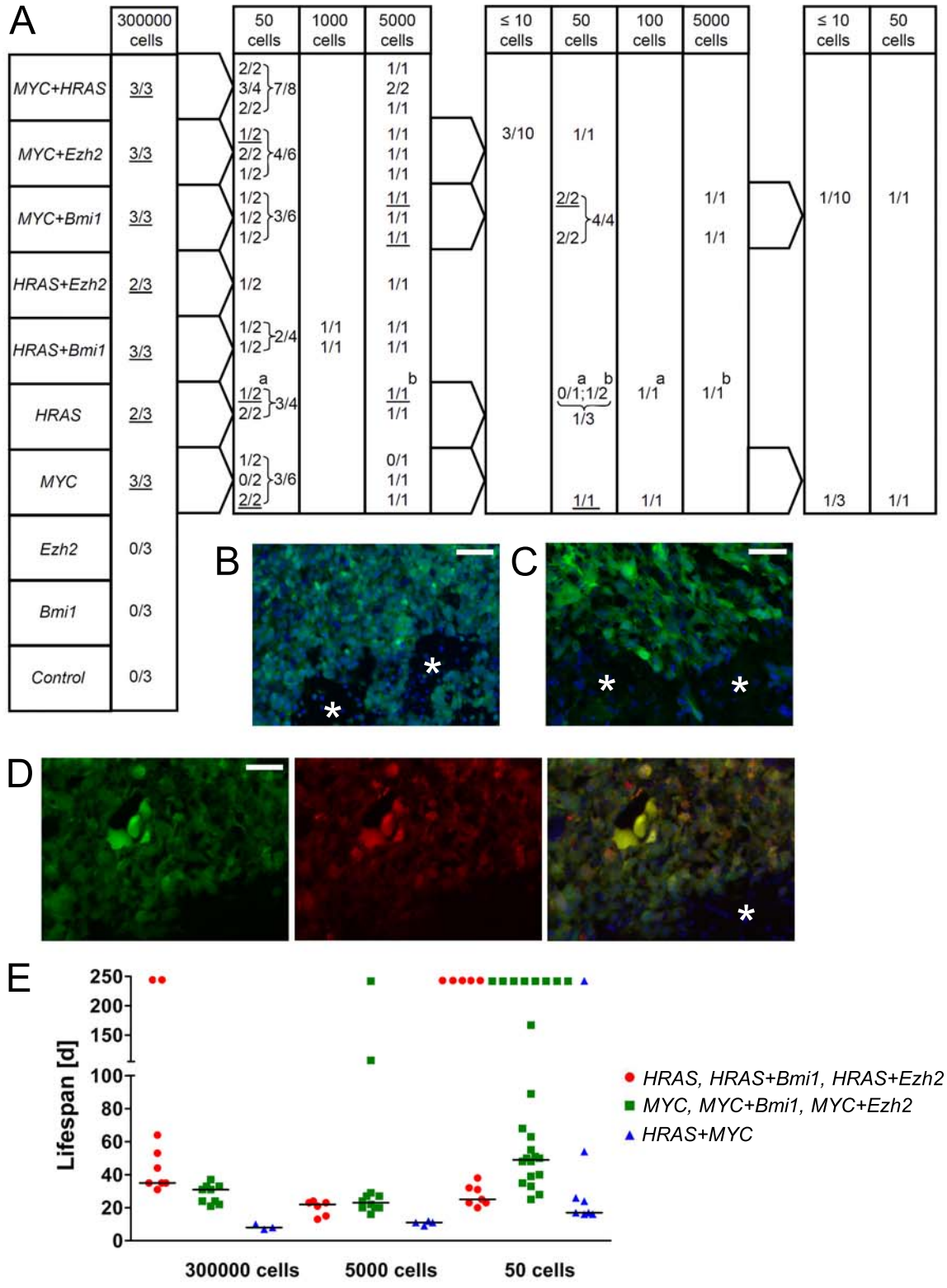

A
300000 cells
50 cells
1000 cells
5000 cells
≤ 10 cells
50 cells
100 cells
5000 cells
≤ 10 cells
50 cells
MYC+HRAS
MYC+Ezh2
MYC+Bmi1
HRAS+Ezh2
HRAS+Bmi1
HRAS
MYC
Ezh2
Bmi1
Control
3/3
3/3
3/3
2/3
3/3
2/3
3/3
0/3
0/3
0/3
7/8
4/6
3/6
2/4
3/4
3/6
4/4
1/10
1/3
B
C
D
E
Lifespan [d]
250
200
150
100
80
60
40
20
0
300000 cells
5000 cells
50 cells
HRAS, HRAS+Bmi1, HRAS+Ezh2
MYC, MYC+Bmi1, MYC+Ezh2
HRAS+MYC

FIGURE 2

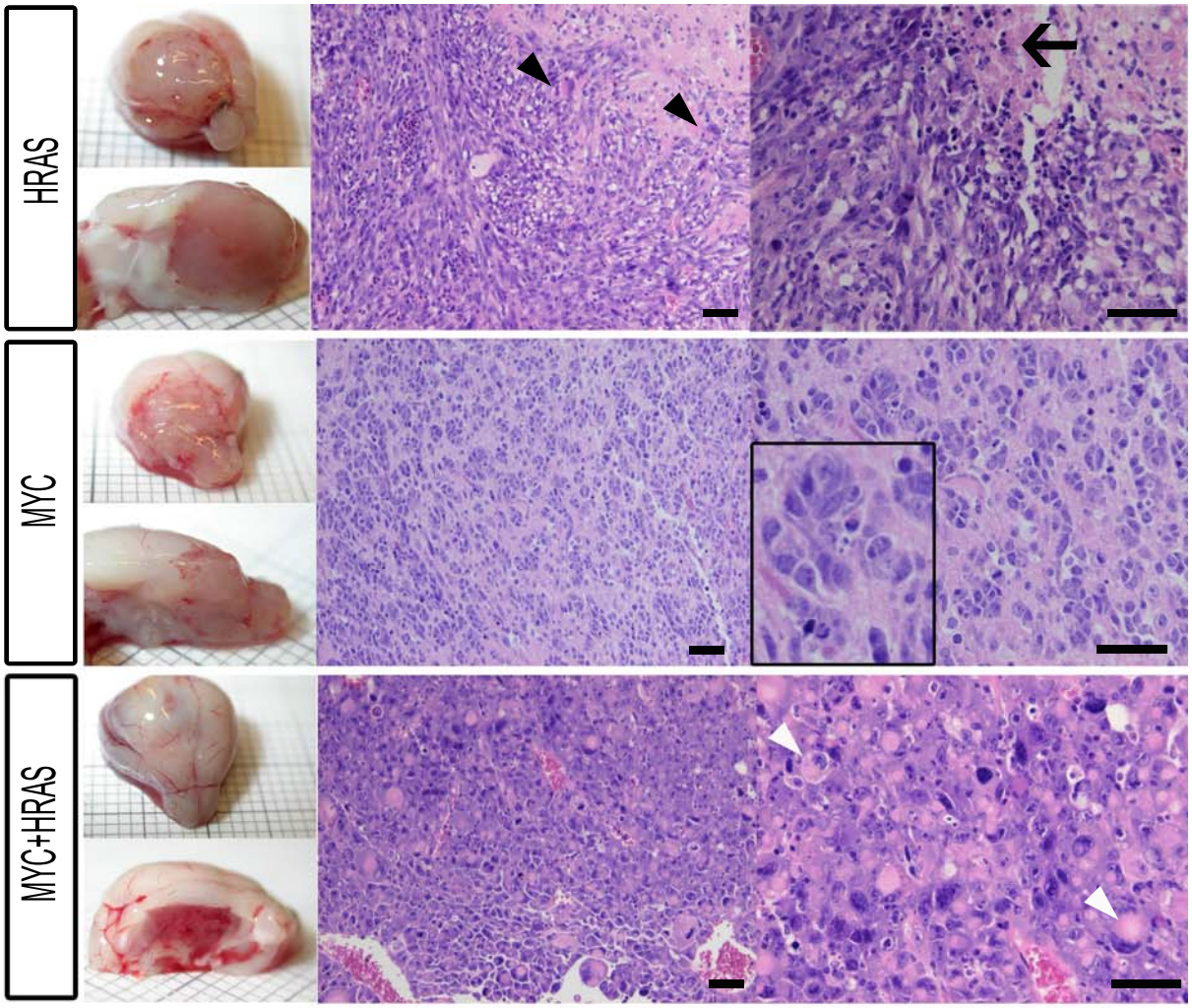
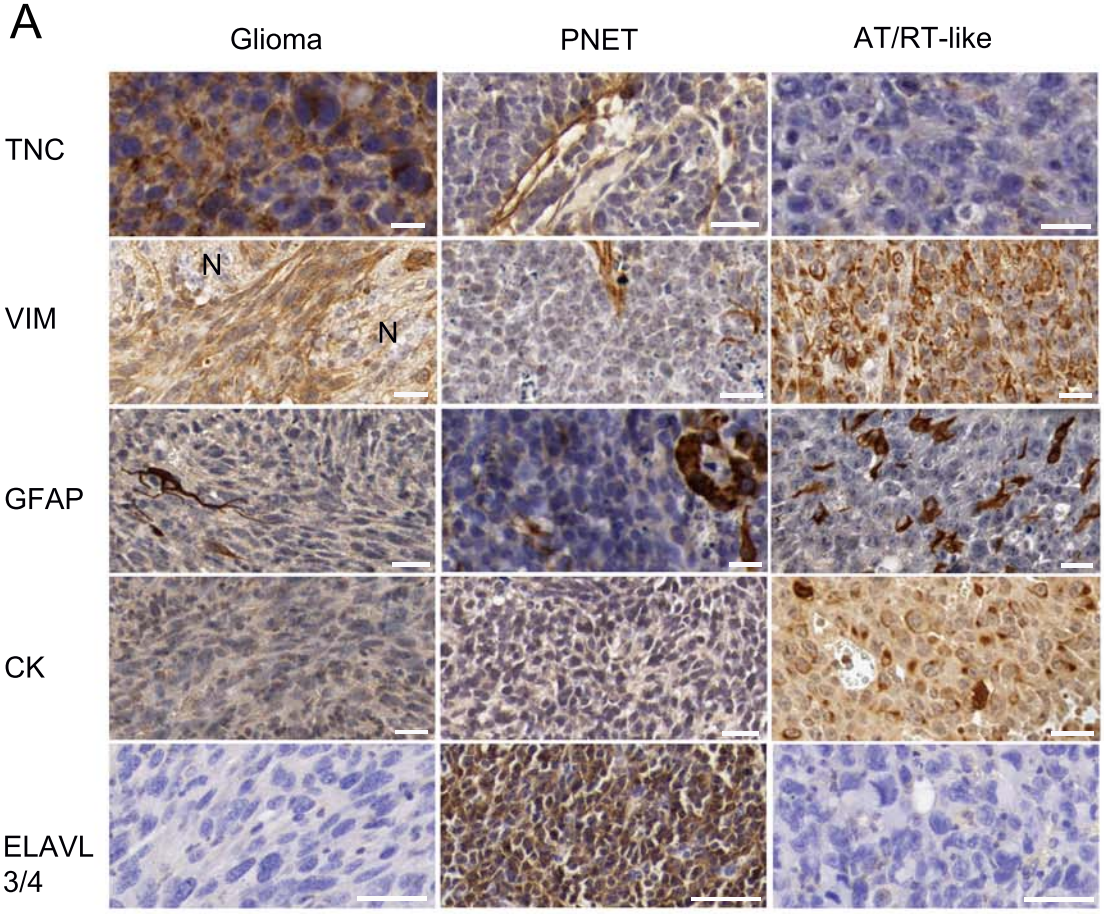


FIGURE 3

A



	TNC	VIM	GFAP	CK	SPP1	ELAVL3/4
Glioma	4/5 +, 1/5 (+)	5/5 +	0/5	0/5	5/5 +	0/5
PNET	0/6	0/6	2/6 [+]	0/6	0/6	5/6 +
AT/RT-like	0/6	6/6 +	3/6 [+]	5/6 [+]	6/6 +	0/6

B

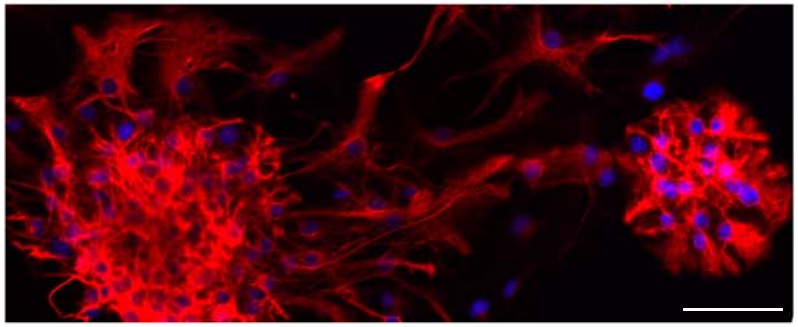


FIGURE 4

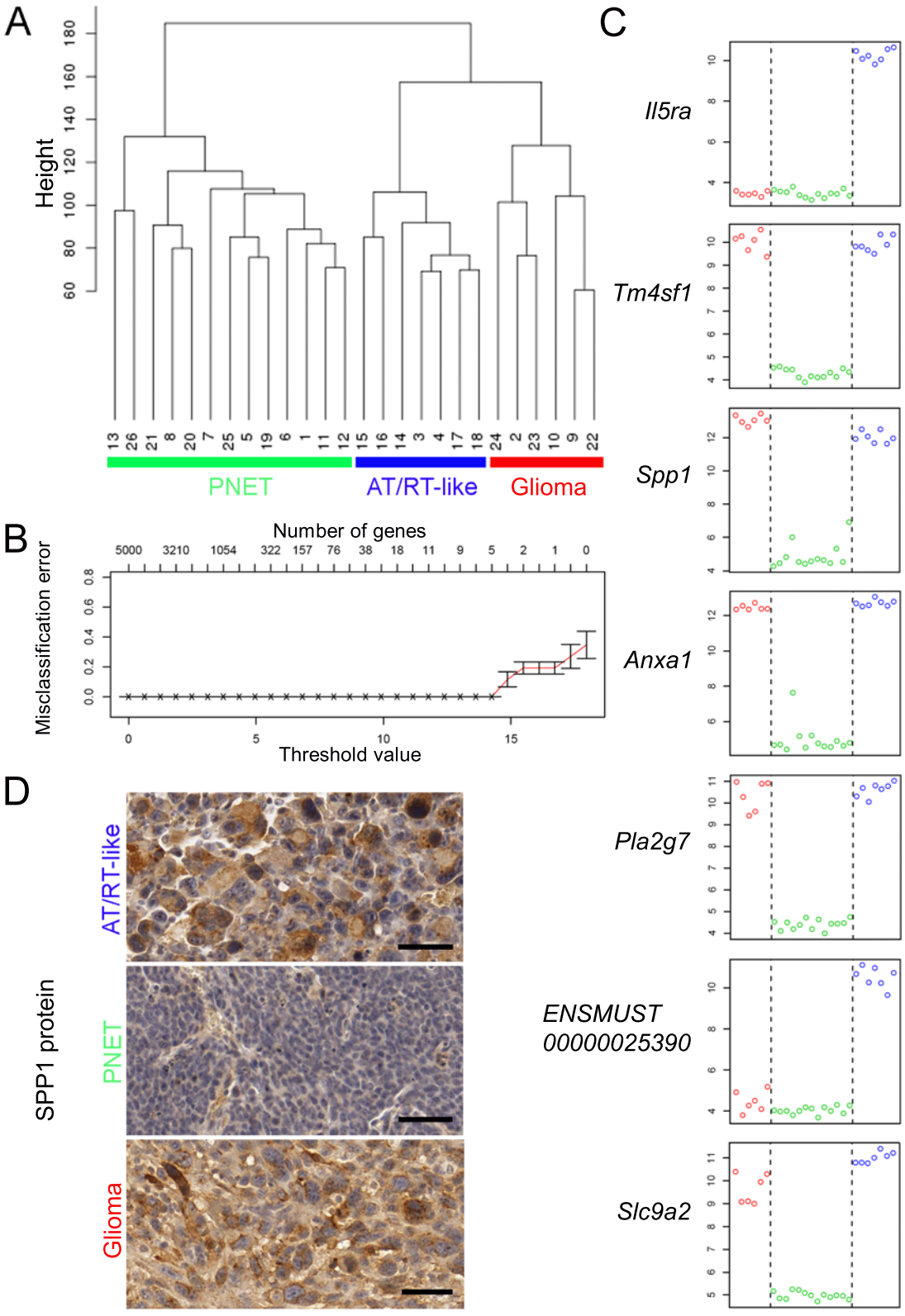
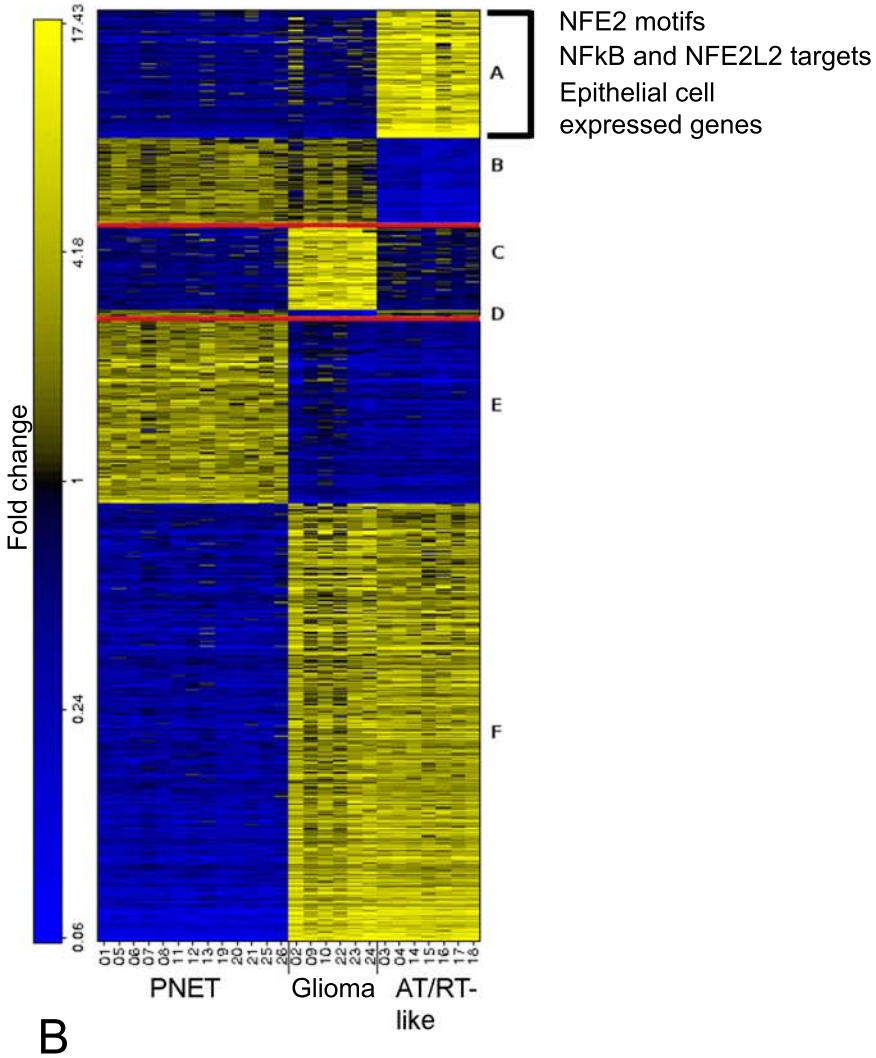

A
Height
PNET
AT/RT-like
Glioma
B
Number of genes
Misclassification error
Threshold value
C
Il5ra
Tm4sf1
Spp1
Anxa1
Pla2g7
ENSMUST
00000025390
Slc9a2
D
SPP1 protein
AT/RT-like
PNET
Glioma

FIGURE 5

A



B

	Gene Set (GS)	GS SIZE	NES	FWER p-value
PNET				
vs AT/RT-like	Medulloblastoma [c]	77	1.72	0.002
vs Glioma	Core ESC-like Module [a]	312	2.00	0.000
	Medulloblastoma [c]	77	1.82	0.000
	Myc Module [b]	461	1.65	0.005
	Mouse ESC-like Module [a]	1214	1.62	0.006
Glioma				
vs AT/RT-like	Malignant Glioma [c]	73	2.02	0.000
	Mouse Adult Tissue Stem Module [a]	694	1.96	0.000
vs PNET	Mouse Adult Tissue Stem Module [a]	694	1.90	0.000
	AT/RT [c]	91	1.72	0.007
AT/RT-like				
vs Glioma	Core ESC-like Module [a]	312	1.94	0.001
	Myc Module [b]	461	1.87	0.002
vs PNET	AT/RT [c]	91	1.67	0.010

FIGURE 6

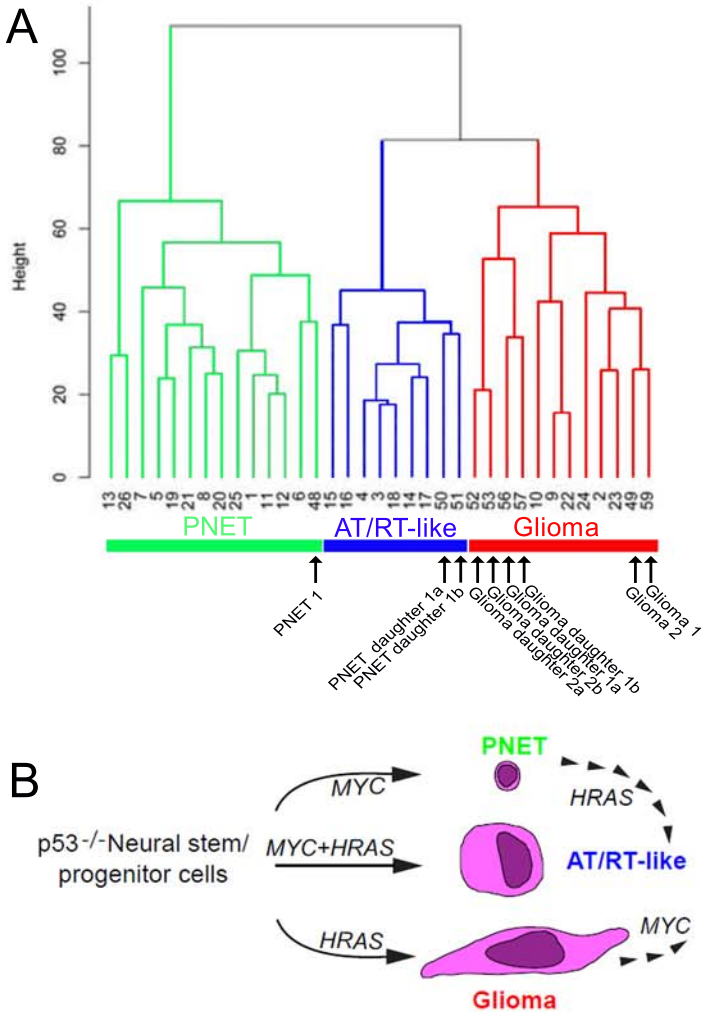

A
Height
0
20
40
60
80
100
13 26 7 5 19 21 8 20 25 1 11 12 6 48 15 16 4 3 18 14 17 50 51 52 53 56 57 10 9 22 24 2 23 49 59
PNET
AT/RT-like
Glioma
PNET 1
PNET daughter 1a
PNET daughter 1b
Glioma daughter 1a
Glioma daughter 2a
Glioma daughter 2b
Glioma daughter 1b
Glioma 1
Glioma 2
B
p53-/-Neural stem/
progenitor cells
MYC
MYC+HRAS
HRAS
PNET
HRAS
AT/RT-like
HRAS
MYC
Glioma

FIGURE 7

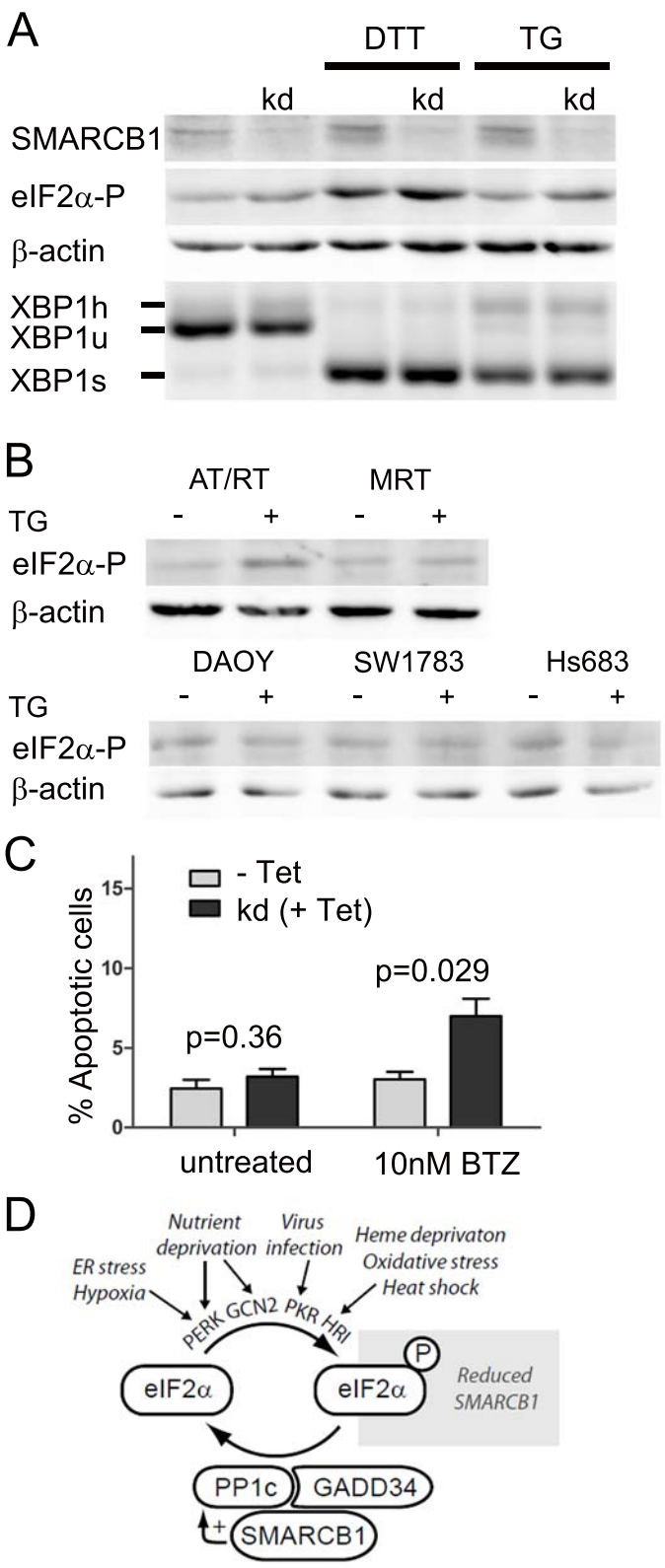

A
DTT
TG
kd
kd
kd
SMARCB1
eIF2α-P
β-actin
XBP1h
XBP1u
XBP1s
B
AT/RT
MRT
TG
- + - +
eIF2α-P
β-actin
DAOY
SW1783
Hs683
TG
- + - + - +
eIF2α-P
β-actin
C
- Tet
kd (+ Tet)
% Apoptotic cells
15
10
5
0
p=0.36
p=0.029
untreated
10nM BTZ
D
Nutrient deprivation
Virus infection
Heme deprivaton
Oxidative stress
Heat shock
ER stress
Hypoxia
PERK GCN2 PKR HRI
eIF2α
eIF2α
P
Reduced SMARCB1
PP1c
GADD34
SMARCB1
+

FIGURE 8

Supplemental Information

Genetic perturbations direct the development of distinct brain tumor types from postnatal neural stem/progenitor cells

Falk Hertwig, Katharina Meyer, Sebastian Braun, Sara Ek, Rainer Spang, Cosima V. Pfenninger, Isabella Artner, Xinbin Chen, Jaclyn A. Biegel, Alexander R. Judkins, Elisabet Englund, Ulrike A. Nuber

Figure S1

Representative macroscopic images (left hand side) and microscopic hematoxylin eosin stained paraffin sections (right hand side) of brain tumors, which developed upon transplantation of neural stem/progenitor cells over-expressing *HRAS* or *MYC* in combination with *Bmi1* or *Ezh2*. Three secondary tumors (first, third and fourth panel) and one primary tumor (second panel) are shown. Arrowheads point to giant cells. Scale bars: 50 µm.

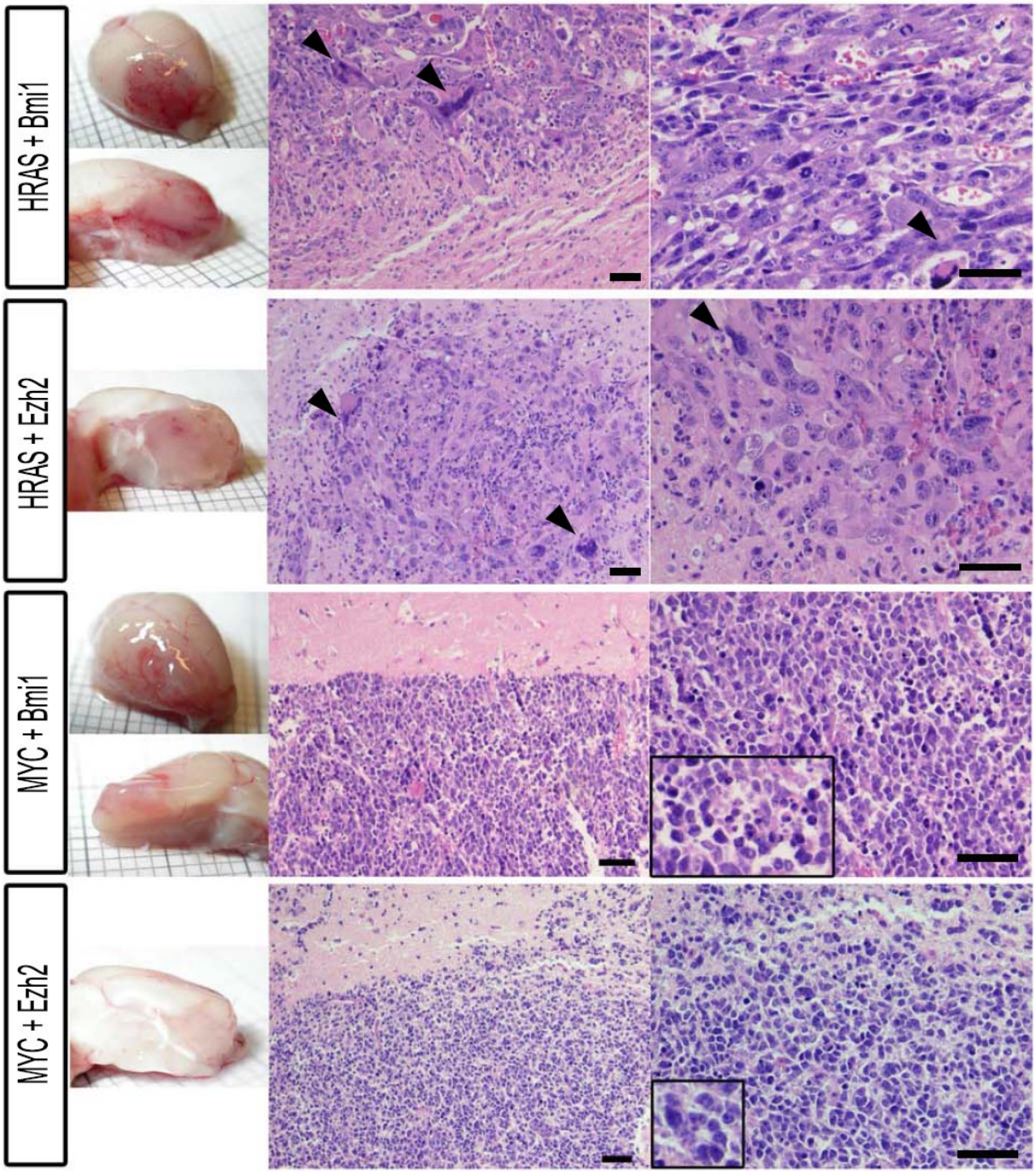
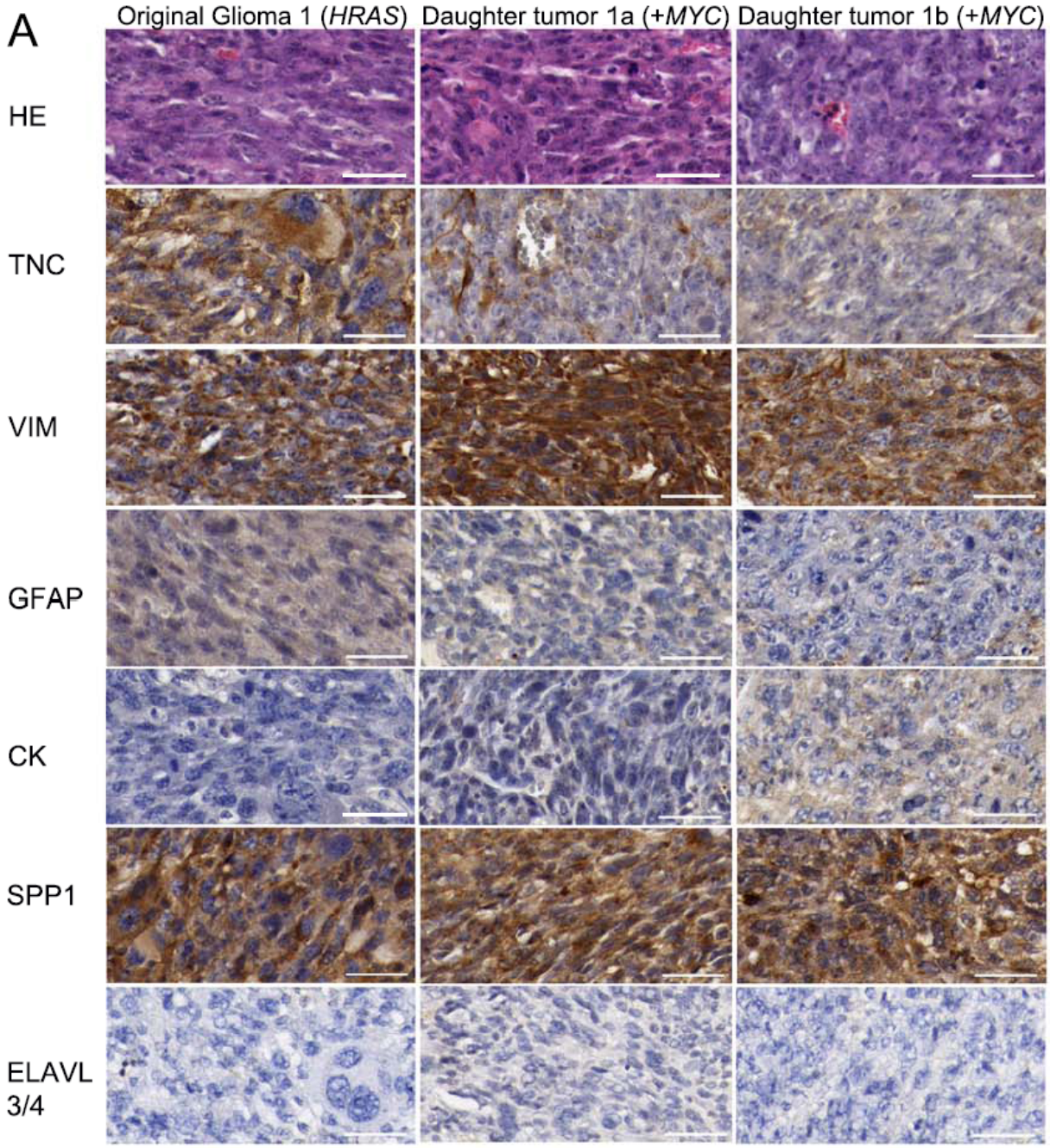
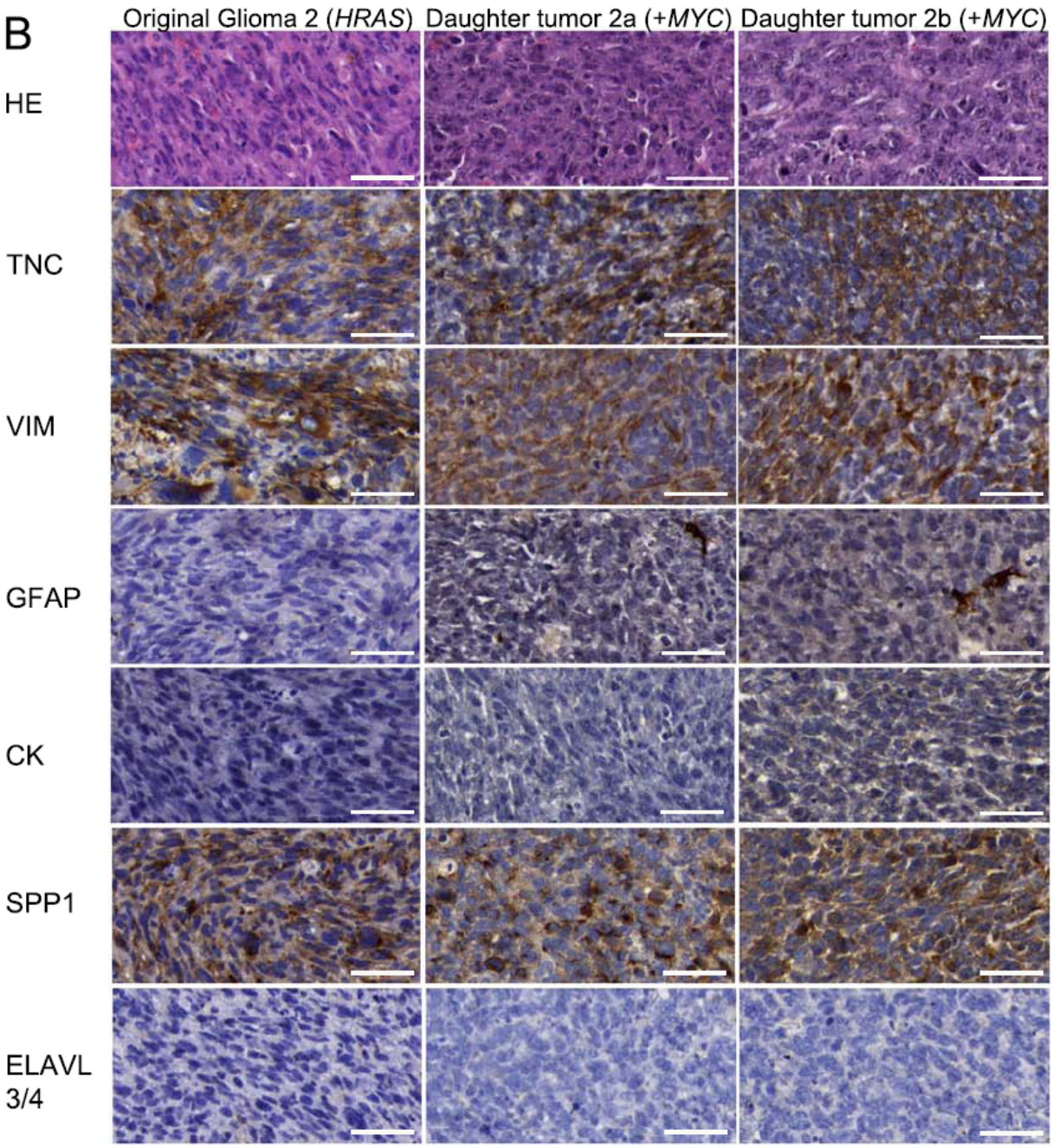


Figure S2

Hematoxylin eosin (HE) stained and immunohistochemistry images of original (mother) and respective daughter tumors which developed upon over-expression of *MYC* in glioma tumors (A,B) or of *HRAS* in CNS PNET tumors (C,D). Paraffin-embedded tumor sections were stained with antibodies against tenascin C (TNC), vimentin (VIM), GFAP, Cytokeratin (CK), SPP1, and ELAVL3/4. [+] Staining of a few tumor cells. V indicates blood vessel. Scale bars: 50 µm.

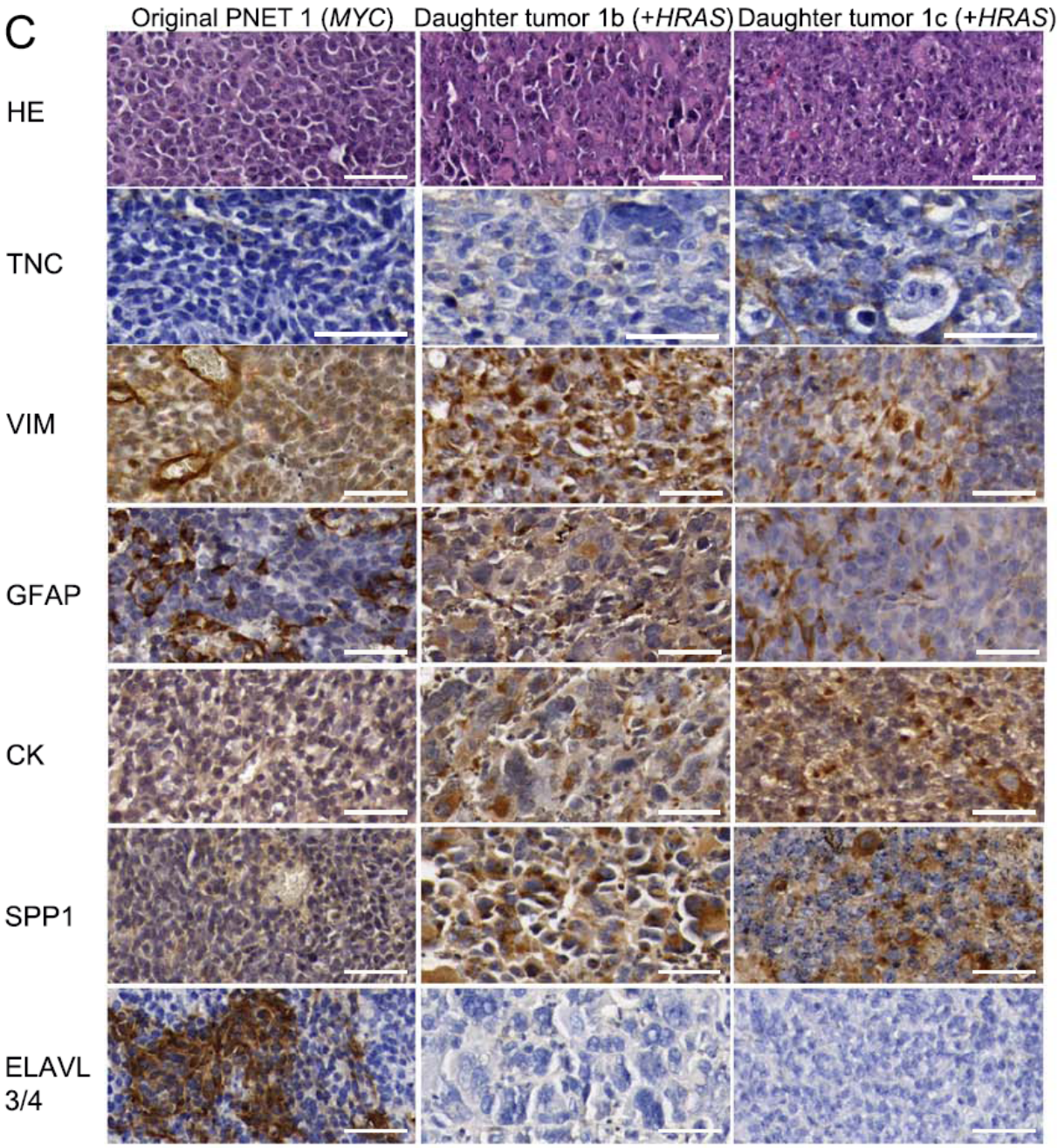


	Original glioma 1	Daughter tumor 1a	Daughter tumor 1b
TNC	+	-	-
VIM	+	+	+
GFAP	-	-	-
CK	-	-	-
SPP1	+	+	+
ELAVL3/4	-	-	-



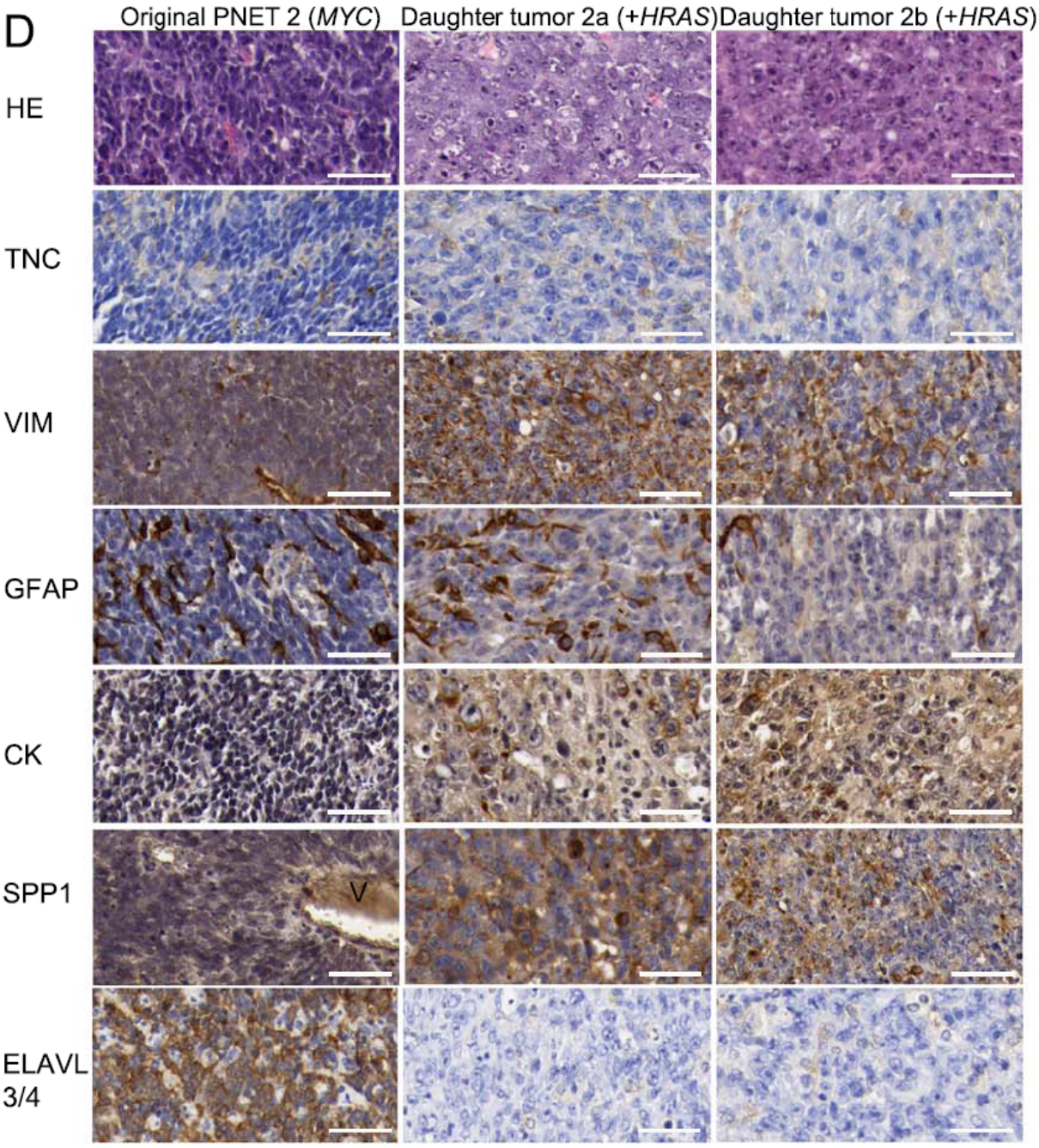
	Original glioma 2	Daughter tumor 2a	Daughter tumor 2b
TNC	+	+	+
VIM	+	+	+
GFAP	-	-	-
CK	-	-	-
SPP1	+	+	+
ELAVL3/4	-	-	-

C



	Original PNET 1	Daughter tumor 1b	Daughter tumor 1c
TNC	-	-	-
VIM	- (only blood vessels +)	+	+
GFAP	[+]	-	[+]
CK	-	[+]	[+]
SPP1	-	+	+
ELAVL3/4	+	-	-

D



	Original PNET 2	Daughter tumor 1b	Daughter tumor 1c
TNC	-	-	-
VIM	- (only blood vessels +)	+	+
GFAP	[+]	[+]	[+]
CK	-	[+]	[+]
SPP1	-	+	+
ELAVL3/4	+	-	-

File S1 (separate file)

Gene sets used in this study.

Table S1

Overview of tumor samples used for microarray experiments. Tumors developed upon transplantation of p53-deficient postnatal neural stem/progenitor cells over-expressing different gene combinations.

Initial combinatorial perturbation experiments			
Gene combination	**Array sample number**	**Transplantation**	**Transplanted cell number**
HRAS + MYC	15	Secondary	50
HRAS + MYC	16	Secondary	50
HRAS + MYC	17	Primary	300000
HRAS + MYC	18	Primary	300000
HRAS + MYC	14	Primary	300000
HRAS + MYC	4	Primary	300000
HRAS + MYC	3	Primary	300000
MYC + Ezh2	1	Primary	300000
MYC + Ezh2	5	Secondary	50
MYC + Ezh2	8	Primary	300000
MYC + Ezh2	19	Primary	300000
MYC + Ezh2	20	Secondary	50
MYC + Bmi1	6	Secondary	5000
MYC + Bmi1	21	Secondary	5000
MYC + Bmi1	7	Secondary	5000
HRAS + Bmi1	22	Primary	300000
HRAS + Bmi1	9	Primary	300000
HRAS	23	Primary	300000
HRAS	2	Secondary	50
HRAS	10	Primary	300000
HRAS	24	Secondary	50
MYC	11	Primary	300000
MYC	12	Primary	300000
MYC	25	Primary	300000
MYC	13	Secondary	50
MYC	26	Tertiary	50
Consecutive perturbation experiments			
Gene combination	**Array sample number**	**Transplantation**	**Transplanted cell number**
HRAS (Glioma 1)	59	Primary	500000
HRAS (Glioma 2)	49	Primary	500000
Additional MYC (daughter 1a)	56	Secondary	500000
Additional MYC (daughter 1b)	57	Secondary	500000
Additional MYC (daughter 2a)	52	Secondary	5000
Additional MYC (daughter 2b)	53	Secondary	5000
MYC (PNET 1)	48	Primary	500000
Additional HRAS (daughter 1a)	50	Secondary	5000
Additional HRAS (daughter 1b)	51	Secondary	5000

Table S2 (separate file):

Six groups of genes (A-F), which show the highest correlation to one of the three tumor types.

Table S3 (separate file):

ER stress relation of Group A genes (higher expressed in AT/RT-like cells as compared to glioma and PNET cells).

Table S4 (separate file):

ER stress relation of genes up-regulated in mouse embryonic fibroblasts upon deletion of the AT/RT tumor suppressor gene *Snf5* and also up-regulated in human AT/RT (SNF5KO_GS_UP genes, Isakoff et al., 2005)

Table S5:

Gene set enrichment analysis to identify molecular signatures of pathways that correlate best to the gene expression profile of the three tumor types (vs = versus). Results with a family wise error rate (FWER) below 0.01 are shown. NES = absolute normalized enrichment score.

	Gene Set (GS)	GS SIZE	NES	FWER p-value
PNET				
vs AT/RT-like	-	-	-	-
vs Glioma	HSA03010_RIBOSOME	64	2.32	0.000
	MRNA_PROCESSING_REACTOME	98	2.00	0.008
Glioma				
vs AT/RT-like	GPCRDB_OTHER	47	2.06	0.000
vs PNET	HSA04510_FOCAL_ADHESION	186	2.22	0.000
	HSA04512_ECM_RECEPTOR_INTERACTION	80	2.19	0.000
	INTEGRIN_MEDIATED_CELL_ADHESION_KEGG	89	2.07	0.000
	PROSTAGLANDIN_SYNTHESIS_REGULATION	27	2.06	0.000
	HSA01430_CELL_COMMUNICATION	119	2.00	0.000
	BLOOD_CLOTTING_CASCADE	18	1.98	0.000
	HSA05222_SMALL_CELL_LUNG_CANCER	83	1.97	0.000
	HSA04060_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	212	1.95	0.001
	CCMX_INTEGRINCELLADH	172	1.94	0.003
	BREAST_CANCER_ESTROGEN_SIGNALING	83	1.92	0.005
	HSA05120_EPITHELIAL_CELL_SIGNALING_IN_HELICOBACTER_PYLORI_INFECTION	65	1.91	0.005
	HSA04640_HEMATOPOIETIC_CELL_LINEAGE	64	1.90	0.005
	HSA04620_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	95	1.90	0.005
AT/RT-like				
vs Glioma	HSA00970_AMINOACYL_TRNA_BIOSYNTHESIS	36	2.18	0.000
	DNA_REPLICATION_REACTOME	41	2.11	0.001
vs PNET	BREAST_CANCER_ESTROGEN_SIGNALING	83	2.08	0.000
	HSA04640_HEMATOPOIETIC_CELL_LINEAGE	64	2.04	0.000
	HSA01430_CELL_COMMUNICATION	119	2.02	0.000
	HSA04060_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	212	2.00	0.002
	PROSTAGLANDIN_SYNTHESIS_REGULATION	27	1.95	0.003
	HSA04620_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	95	1.93	0.005
	BLOOD_CLOTTING_CASCADE	18	1.92	0.006

Supplemental experimental procedures

Animal procedures

In the pilot study, female and male 4-8 weeks old wild-type C57Bl/6 mice were used for transplantations, for the main study female 4-8 weeks old wild-type C57Bl/6 mice received intracranial cell transplants. Mice were anesthetized by intraperitoneal injection of ketamine/xylazine solution. Cell concentrations were determined with CASY cell counter (CASY-TT; Innovatis; Reutlingen, Germany) or in case of lower cell numbers by a Hemacytometer. In case of 1000 or more transplanted cells, cells were harvested by centrifugation and resuspended in 3 µL PBS. In case of lower transplanted cell numbers, cells were injected in a 2-5 µl suspension in neurosphere medium. Injections were performed with 10µL gastight Hamilton syringes (blunt end 26G needles; Hamilton Bonaduz AG, Switzerland) into the right frontal brain lobe (2 mm lateral/1 mm anterior to the bregma, and 3 mm depth).
Transplanted mice of the pilot study were monitored for 6 months, mice of the main study for 8 months. Animals which showed symptoms such as ataxia, reduced movements or seizures were sacrificed and brains were isolated. Macroscopic brain images were taken with a Nikon Coolpix 4500 camera. A part of the tumor tissue was removed to isolate tumor cells by FACS. The remaining brain tissue was used for paraffin and cryosections. All animal procedures were performed with consent from the ethical committee at Lund University.

Viral vectors and transduction

Human *c-Myc* was kindly provided by Rogier Versteeg, human V^{12} *Ha-Ras* cDNA was obtained by PCR using genomic DNA from RasB8 mice (kindly provided by Abhijit Guha; Ding et al.). To generate mouse *Bmi1*, *Ezh2*, and *FoxM1* cDNA, RNA was isolated from neurosphere cultures that were established from postnatal wt mouse LVW tissue, reverse-transcribed and used as a template for PCR with gene-specific primers. $6x10^6$ cells of the ecotropic retroviral packaging cell line EcoPack2-293 (Clontech) were plated in 12 ml DMEM medium per T75 CellBind tissue culture flasks (Corning). The DMEM medium contained 1g/L glucose (Lonza), 10% FBS, 1 mM sodium pyruvate (both from Biochrom), 4 mM Glutamine, 100 units/mL Penicillin/Streptomycin (Invitrogen), and 2.5µg/mL Amphotericin B (PAA). 18-24h later, the medium was changed to transfection medium (DMEM medium as above with 25 µM chloroquine), and cells were transfected by the calcium phosphate method (as described in Mangassarian et al. 1999). After 16h, the medium was removed, cells were briefly treated with 15% glycerol in HeBS (25 mM HEPES, 140 mM NaCl, 0.75 mM Na_2HPO_4, all from Sigma), and grown in neurosphere medium for 48h. Cell supernatant was harvested, filtered by passing through a 0.45 µm PVDF membrane filter (Millipore) to remove remaining EcoPack2-293 cells and debris, and concentrated by centrifugation (2h, 20000x g). Viral pellets were resuspended in a final volume of 1 mL neurosphere medium containing 4 µg/mL polybrene (Sigma Aldrich) and applied to $3x10^6$ dissociated neurosphere cells per gene combination in 15 mL Falcon tubes for 4h at 37°C and 5% CO_2. The neurosphere cells were then harvested by centrifugation, resuspended in 15 mL fresh neurosphere medium without polybrene, seeded into T75 flasks with UltraLowAttachment surface (Corning), kept at 37°C and 5% CO_2, and FACS sorted after approximately 7 days.

RT-PCR

For cDNA synthesis, 2 µg of total RNA were incubated with 5 µl 10 mM dNTPs (Fermentas, Burlington, Canada) and 2.5 µL random primers (500ng/µL) in a 35 µl reaction volume at 70°C for 5 min followed by a short incubation on ice and reverse transcription at 37°C for 60 min upon addition of 200 U M-MLV reverse transcriptase (Promega, Madison, WI), 0.5 U RNAsin (Promega), and 10 µl 5x M-MLV reaction buffer (Promega) to a final reaction volume

of 50 µL. 2 µL of this solution were used as template in a 50 µL polymerase chain reaction (PCR) with 0.4 µM of the forward and reverse primers.
Reaction conditions for the amplification of oncogene sequences were: 35 cycles (94°C for 45 sec, 55°C for 45 sec, 72°C for 45 sec). The following forward primers were used:
mEzh2_end_for (ATGGTGACCACAGGATAGGC)
mFoxM1_end_for (TTTCAGCCAACCGTTCTCTC)
Ha-Ras_end_for (GGATGCCTTCTACACGTTGG)
H_cMyc_end_for (AAAGGCCCCCAAGGTAGTTA), and
Bmi1ERfusion for (AGGTGTTCCCTCCACCTCTT).
CMMP 3' end rev (CGGATCCCCCTGATCCTC) was used as a reverse primer.
For the XBP1 PCR, primers hXBP1_Fw (GGAGTTAAGACAGCGCTTGG) and hXBP1_Rv (ACTGGGTCCAAGTTGTCCAG) were used. Reaction conditions were: 30 cycles (94°C for 45 sec, 61°C for 45 sec, 72°C for 90 sec).

Immunohistochemistry

Paraffin sections were rehydrated, boiled in citrate buffer (10 mM Sodium citrate, 0.05% Tween-20; pH6.0) for antigen retrieval, incubated in 0.35% H_2O_2, and blocked with 5% donkey serum/PBS. Primary antibodies were applied in PBS for 16h at 4°C (PBS only served as negative control); Biotin-conjugated secondary antibodies (1:500; Jackson Immunoresearch, West Grove, PA) were applied for 90 mins at room temperature. The R.T.U vectastain kit and DAB peroxidase substrate were used according to the manufacturer's instructions (VectorLabs, Burlingame, CA). Finally, the tissue sections were counterstained with Haematoxylin, dehydrated, and embedded in Entellan. The following primary antibodies were used: anti-BAF47 (SMARCB1/SNF5; 1/100; BD Biosciences, San Diego, CA), anti-pan-Cytokeratin (1:100; DAKO, Glostrup, Denmark), anti-glial fibrillary acidic protein (GFAP; 1:500, DAKO), anti-human neuronal protein HuC/HuD (ELAVL3/4; 1:400; Molecular Probes/Invitrogen, Carlsbad, CA), anti-Tenascin (1:400; kind gift of A. Faissner; Ruhr-University, Bochum, Germany), anti-Osteopontin/SPP1 (1:400; R&D Systems, Minneapolis, MN), anti-Vimentin (1:400; Progen Biotechnik, Heidelberg, Germany). Images of paraffin sections were taken with an Olympus BX50 camera or ScanScope scanner. Images of 10 µm cryosections were taken with an Axiovert 200M microscope using AxioVision 4.5 software (Zeiss).

Western blot analysis

Cells were harvested at 50-70% confluency, pelleted by centrifugation, and dissolved in RIPA buffer (10 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 100 mM NaCl) supplemented with protease inhibitors (2mM PMSF and 1x Complete Mini, Roche). Supernatants were collected, protein concentrations were determined using the BCA assay (Pierce/Thermo Scientific), and proteins were separated in a 10% SDS-polyacrylamide gel. Western blotting followed standard protocols using nitrocellulose membranes (Amersham/GE Healthcare). Signal intensities were analyzed with ImageJ (http://rsb.info.nih.gov/ij/).

Culture of human cell lines

The CHLA-02-ATRT cell line was obtained from ATCC and kept in neurosphere medium (see experimental procedures). The human malignant rhabdoid tumor cell line LM (provided by Rupert Handgretinger, Tuebingen, Germany) was kept according to Versteege et al. (Nature 1998). DAOY medulloblastoma cells (provided by Michael Grotzer, Zurich, Switzerland) were kept in EMEM (Lonza) supplemented with 10% FBS (Biochrom), 2 mM glutamine, 100 units/mL Penicillin/Streptomycin (Invitrogen), and 2.5 µg/mL Amphotericin B (PAA). SW1783 and Hs683 glioma cell lines (provided by Matthias Simon, Bonn, Germany) and were kept in DAOY medium with 1 mM sodium pyruvate, MEM non essential amino

acids, and MEM vitamin solution (all from Invitrogen). MCF7-SNF5-KD#73 cells (Xu et al., 2010) were cultured in DMEM (4.5g/L glucose, Invitrogen), 10% FBS (Tet System Approved, Clontech), 2 mM glutamine, and 100 units/mL Penicillin/Streptomycin (Invitrogen). For the knockdown of SMARCB1, tetracycline (1 µg/mL) was added on day 1 and 3, and cells harvested at day 4. At day 4, cells were treated with DTT (5 mM) or Thapsigargin (5 µM). All cells were kept in T75 tissue culture flasks (Techno Plastic Products, Switzerland).

Apoptosis assay

500,000 MCF7-SNF5-KD#73 cells (Xu et al., 2010) were plated into T75 tissue culture flasks (Techno Plastic Products, Switzerland). For knockdown experiments, tetracycline (1 µg/mL) was added on day 1 and day 3. In case of Bortezomib-treated cells, the drug (final concentration 10 nM) was added for 12 hours on day 4 (at 70-85% confluency). Cells were carefully washed with PBS and dissociated by trypsinization. Aliquots of single cell suspensions containing $1x10^5$ cells in 100 µL PBS were stained with AnnexinV-APC and 7AAD according to the manufacturer's instructions (BD Biosciences) for 15 min at RT. Subsequently, 400 µL of AnnexinV binding buffer was added (10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$), cell suspensions were placed on ice and immediately analyzed with a FACSCalibur flow cytometer (BD Biosciences). The data analysis was performed using the FlowJo software (Tree Star). For statistical analyses, an unpaired t-test was conducted using Prism 5.0 (GraphPad, LaJolla, CA, USA).

Identification of novel BMI1 targets in neural stem/progenitor cells

Falk Hertwig[1,2,3] , Sebastian Braun[1,3], Ulrike A. Nuber[1]

[1] Lund Strategic Center for Stem Cell Biology, Lund University, 22184 Lund, Sweden
[2] Section Biology, Chemistry and Pharmacy, Freie Universität Berlin, 14195 Berlin, Germany
[3] These authors contributed equally

Correspondence: ulrike.nuber@med.lu.se, phone: +46 46 22 21563, fax: +46 46 22 23600

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ABSTRACT

Bmi1 was originally identified as a gene that contributes to the development of mouse lymphoma by inhibiting MYC-induced apoptosis via *Ink4a* and *Arf* suppression. BMI1 is a polycomb group protein and acts as a transcriptional repressor by means of chromatin changes. Knock-out and knock-down studies have shown that BMI1 is required for the maintenance of normal postnatal neural stem cells and of brain tumor cells and this function partly relies on the repression of *Ink4a*/*Arf*. Here we show that *Bmi1*-over-expressing postnatal neural stem/progenitor cells display increased self-renewal and survival, however, intracranial transplantations of these cells fail to initiate tumor growth, suggesting that BMI1 rather plays a role as a facilitator of other transforming events. Although the protein is supposed to bind to a large number of genomic regions, the few direct BMI1 target genes described so far likely do not account for the full range of BMI1-mediated neural stem cell effects. We therefore sought to identify novel direct BMI1 targets. Microarray gene expression analysis revealed several genes which are down-regulated in *Bmi1*-over-expressing neurosphere cells, and we show that BMI1 binds to genomic regions of four of them: *Ndn*, *EphA7*, *Trp53bp2*, and *Rps6ka6*. All of these novel BMI1 candidate targets are implied to affect stem cell functions.

INTRODUCTION

Bmi1 (B cell-specific Mo-MLV integration site 1) was identified as a gene cooperating with *Myc* in the generation of B-lymphoid tumors (Haupt et al., 1991; van Lohuizen et al., 1991). BMI1 was further characterized as a polycomb group gene product that acts as a transcriptional repressor as a part of the Polycomb Repressor Complex 1 (PRC1), which regulates chromatin changes (Valk-Lingbeek et al., 2004). *Bmi1* over-expression enables immortalization of murine fibroblasts (Jacobs et al., 1999b) and the gene cooperates with *HRAS* to induce transformation of human mammary epithelial cells (Datta et al., 2007). Its transformation-supportive role is mainly due to the repression of the tumor suppressors INK4A and ARF, which are cell cycle inhibitors and can induce senescence or apoptosis (Jacobs et al., 1999a; Jacobs et al., 1999b; Lowe and Sherr, 2003). Moreover, elevated levels of BMI1 are found in hematopoietic malignancies (Bea et al., 2001), brain tumors (Leung et al., 2004; Bruggeman et al., 2007; He et al., 2009), as well as a variety of epithelial cancers (Voncken et al., 2003; Song et al., 2006; Tateishi et al., 2006; Wang et al., 2008; Song et al., 2010).

In addition to tumor development in connection with *MYC* or *HRAS*, *BMI1* is essential for the maintainance of established human brain tumors (Abdouh et al., 2009). Stable knock-down of *BMI1* expression in cultured cancer stem cell-enriched glioblastoma cells impairs their tumorigenic properties *in vivo* (Abdouh et al., 2009).

Bmi1 deficiency depletes the postnatal stem cell pools of the murine hematopoietic and nervous system (Molofsky et al., 2003; Park et al., 2003). The impaired neural stem cell self-renewal and proliferation in *Bmi1*-deficient mice is largely due to derepressed *Ink4a*/*Arf* levels (Molofsky et al., 2003; Bruggeman et al., 2005; Molofsky et al., 2005). However, it could also be shown that *Bmi1* affects self-renewal of neural stem cells and glioma cells in an *Ink4a*/*Arf*-independent (Bruggeman et al., 2007) manner. Applying shRNA-mediated knock-down of *Bmi1*, Fasano et al. (2007) could extend the functional network of *Bmi1*-mediated cell cycle regulation with the identification of *Cdkn1a* (encoding the cell cycle inhibitor $p21^{CKI}$) as a downstream target of BMI1 (Fasano et al., 2007).

The cooperation of *BMI1* with *MYC* and *HRAS* in oncogenic transformation and its implication in neural stem cell self-renewal raises the question whether abnormally high *Bmi1* expression levels in murine neural stem cells can render these cells tumorigenic. Fasano et al. (2009) and He et al. (2009) demonstrated that the over-expression of *Bmi1* in neural stem/progenitor cells leads to higher self-renewal rates *in vitro* and He et al. reported a slightly increased *in vivo* cellular expansion. The mouse model used by He et al. (2009) also suggests that the *Bmi1* transgene under the Nestin promoter is not sufficient to initiate brain tumor growth.

In this study, we show that over-expression of *Bmi1* confers a strong advantage to neural stem/progenitor cells *in vitro*, promoting their self-renewal, cellular expansion and survival, but it does not induce tumorigenesis, as assessed by intracranial transplantation experiments.

Since it seems unlikely that the few hitherto identified direct BMI1-regulated genes (*Cdkn2a*, *Cdkn2b* Bracken et al., 2007; *Cdkn1a*, *HOXC13* Abdouh et al., 2009) account for the full range of BMI1-mediated effects, we sought to identify novel direct targets of this transcriptional repressor. Here we show that various genes are down-regulated in *Bmi1*-over-expressing neurosphere cells and we demonstrate a binding of BMI1 to genomic regions of four of them: *Ndn*, *EphA7*, *Trp53bp2*, and *Rps6ka6*.

Our results strengthen the hypothesis that high BMI1 levels promote stem cell properties but are not sufficient for oncogenic transformation of neural stem/progenitor cells. In addition, we expand the BMI1 network with the identification of four novel direct targets, all of which are implicated in cellular functions relevant for stem cell maintenance.

RESULTS

***Bmi1* over-expression enhances the *in vitro* expansion of neurosphere cells but does not lead to their transformation into tumorigenic cells**

To investigate the effect of *Bmi1* over-expression on neural stem cell growth and tumorigenic potential, lateral ventricle wall (LVW) stem/progenitor cells of 4-6 week-old mice were grown as neurospheres and transduced with bicistronic retroviral constructs enabling the co-expression of a *Bmi1* transgene and eGFP. Two *Bmi1* over-expression constructs (pCMMP-Bmi1 and pCMMP-Bmi1-FLAG) and an empty vector (EV) control construct were used in this study (Supplementary Figure S1). After FACS purification, the positive transductants were subjected to a neurosphere (NSP) assay under clonal conditions (1 cell/µL). We assayed self-renewal properties by determining the frequency of neurosphere initiation (NSI), and cell expansion by measuring cell counts at each passage (Figure 1A, B). An increased neurosphere initiation rate was observed in cultures over-expressing *Bmi1* compared to EV controls. Neurospheres generated by *Bmi1*-over-expressing cultures were also significantly larger than EV controls (Figure 1C, D; neurosphere diameters of EV: 66.31 µm±2.1 µm, pCMMP-Bmi1: 105.0 µm±2.9 µm, pCMMP-Bmi1-FLAG: 125.2 µm±2.2 µm; mean±SEM), indicating that in addition to a higher neurosphere number, neurospheres contained more cells. In line with these findings, growth curves showed an increase in cell number upon *Bmi1* over-expression (Figure 1B). We deducted from the growth curve that Bmi1-over-expressing cultures had an average doubling time of 28 h (pCMMP-Bmi1 and pCMMP-Bmi1-FLAG) compared to 44 h doubling time of EV

control cultures. These results demonstrate that neural stem/progenitor cell expansion under these *in vitro* conditions can be greatly enhanced by the over-expression of *Bmi1*.

We next sought to investigate if the observed proliferative advantage of *Bmi1*-over-expressing cells was accompanied by tumorigenic properties. Neurosphere cells, which were transduced with *Bmi1* over-expression vectors or the EV control construct were transplanted intracranially into both syngeneic and immunocompromised mice (Figure 1E), which were monitored for 5-6 months. Tumor formation was observed in mice receiving 300,000 cells of the mouse glioma cell line GL261 cells as positive control, but not in animals transplanted with 300,000 *Bmi1*-over-expressing neurosphere cells or neurosphere cells transduced with empty vector. These data indicate that abnormally high levels of BMI1 can confer a growth advantage to neurosphere cells in culture, but do not lead to tumor development *in vivo*.

Bmi1 over-expression promotes proliferation and increases survival in neurosphere cells

Cultured cells are known to express increased *Ink4a*/*Arf* levels, as a result of "cell culture stress", leading to apoptotic events and the inhibition of cell cycle progression *in vitro* (Sherr and DePinho, 2000). Thus, the growth advantage resulting from *Bmi1* over-expression in culture could be explained by a BMI1-mediated repression of increased *Ink4a*/*Arf* levels (He et al., 2009).

We next investigated whether over-expression of *Bmi1* has an effect on proliferation and cell survival of neurosphere cells in culture. Neurosphere cultures transduced with either *Bmi1* over-expression constructs or the EV control were analyzed for apoptotic and dead cells at an early passage of the neurosphere assay (passage 3-4). Dissociated single cells were stained with 7AAD and Annexin V to visualize dead and apoptotic cells, respectively, and analyzed by flow cytometry. Supplementary Figure S2A shows the gating strategy used to identify living (quadrant 3; $7AAD^-/AnnexinV^-$), early apoptotic (quadrant 4; $7AAD^-/AnnexinV^+$) and dead cells (quadrant 2; $7AAD^+/AnnexinV^+$). Counted events for the respective gates are plotted in Figure 2A. We found a significant increase of the live cell population ($7AAD^-/AnnexinV^-$) upon *Bmi1* over-expression compared to EV controls. In addition, we found reduced dead cell counts, indicating that *Bmi1* over-expression enhances survival in neurosphere cultures.

To assess proliferation, we applied a dye retention assay. Neurosphere cells were labeled with the fluorescence dye PKH26, which is distributed to daughter cells, decreasing its fluorescence intensity per cell division. Therefore, the loss of fluorescence intensity is greater in faster dividing cells. *Bmi1* over-expression and EV control cultures were labeled with the red-fluorescing dye PKH26 after neurosphere dissociation and cultured for six days under clonal conditions. Consecutively, PKH26 levels per single cell were measured by flow cytometry. Supplementary Figure S2B shows histograms of the PKH26 fluorescence intensities, measured in *Bmi1*-over-expressing and EV cultures after six days *in vitro*. Mean fluorescence intensities (MFI) are depicted in Figure 2B. We found a significant decrease of MFI levels in *Bmi1*-over-expressing cells indicating an increased cell proliferation.

Taken together, our data indicate that the observed *in vitro* growth advantage of *Bmi1*-over-expressing cells - resulting in an increased self-renewal and cell expansion - is due to an increased proliferation and decreased apoptosis.

Gene expression and ChIP analyses reveal an up-regulation of ES cell signature genes and lead to the identification of novel BMI1 target genes

To gain a molecular understanding how BMI1 induces proliferation and represses apoptotic events in neural stem/progenitor cells in culture, we compared the gene expression profile of neurosphere cells over-expressing *Bmi1* with their respective EV controls using Affymetrix Gene ST1.0 arrays. A gene set enrichment analysis of the overall gene expression data obtained for *Bmi1*-over-expressing and EV control cells was performed. We applied gene expression signatures previously identified in a comparison of different adult neural stem cells, embryonic stem (ES) cells and tumor cells (Pomeroy et al., 2002; Ben-Porath et al., 2008; Wong et al., 2008; Kim et al., 2010). Due to the relatively small number of gene sets applied (25 gene sets; see Supplementary File S1), we only considered gene sets with a very stringent FWER value of < 0.01 as relevant. Two out of five ES cell signature gene sets fulfilled this criterion (Supplementary Table S1) and showed an enrichment in the *Bmi1* over-expression profile. These two gene sets were earlier identified in comparative analyses, comprising genes that were over-expressed in ES cell samples compared to cells from other tissues (ES EXP1 - (Ben-Porath et al., 2008)) or to sets of differentiated cells and other somatic stem cells (CORE ES CELL MODULE - (Wong et al., 2008)). The other three ES cell gene sets were not found as highly enriched in our *Bmi1* data set. This might be due to the fact that they contain only a subset of ES cell enriched genes, or represent defined sets of ES cell-specific transcription factor target genes (see Supplementary Table S1). The enrichment of the gene sets' components is shown in Figure 3A. Although excluded by the chosen FWER threshold, a gene set characterizing adult stem cells was found enriched in the EV control expression profile in comparison to *Bmi1*-over-expressing cells (Supplementary Table S1). These results indicate that *Bmi1* over-expression promotes a more immature stem cell phenotype in neurosphere cultures.

To identify differentially expressed genes from the microarray data, we applied stringent criteria (p2val <0.01; mean logarithmic differential expression levels >1). 300 probe sets met these criteria, 200 of which showed down-regulation of the corresponding transcripts in *Bmi1*-over-expressing cells, and 100 transcripts were up-regulated (Supplementary File S2). We next focused on the identification of BMI1 target genes, which could contribute to the BMI1-mediated regulation of apoptosis, proliferation and self-renewal. Figure 3B shows some of the genes repressed by BMI1, which we considered relevant. Imprinted genes were included since they might be mechanistically linked to polycomb-mediated gene silencing. A direct binding of BMI1 to selected genomic regions of down-regulated genes was investigated by chromatin immunoprecipitation (ChIP, Figure 4). Primer pairs spanning the BMI1-bound *Ink4a* promoter region (Matheu et al., 2005; Bracken et al., 2007) were used as positive control. A binding of BMI1 to genomic regions of four novel target genes was detected (Figure 4): *Ndn*, *EphA7*, *Rps6ka6*, and *Trp53bp2*. *Ndn*, a paternally imprinted gene, is discussed as potential tumor suppressor (Chapman and Knowles, 2009); *EphA7* encodes a signaling receptor involved in regulating apoptosis in neural progenitors (Depaepe et al., 2005). The tumor suppressor gene *Rps6ka6* regulates stress-dependent and replicative sencescence (Lopez-Vicente et al., 2009) and *Trp53bp2* is known as an enhancer of pro-apoptotic transactivation functions of p53 (Vives et al., 2006).

Taken together, our data obtained by comparing gene expression profiles of *Bmi1*-over-expressing neurosphere cells with empty vector control cells indicate that increased BMI1 levels promote the

expression of certain ES cell signature genes and lead to the repression of four novel direct targets, which are implicated in cellular functions relevant for stem cell maintenance.

DISCUSSION

Long-term self-renewal capacity is a hallmark of normal and cancer stem cells. In contrast to the self-renewal of normal stem cells, which is strictly regulated according to physiological demand, the self-renewal of tumor stem cells is poorly controlled. An important task in the field of regenerative medicine and cancer research is to understand the self-renewal programs of normal and cancerous stem cells and accumulating evidence indicates that they are regulated by similar molecular networks, which involve proto-oncogenes and tumor suppressor genes (Pardal et al., 2005; Zheng et al., 2008; Wang et al., 2009). BMI1 has been identified as a protein implicated in the self-renewal of normal hematopoietic and neural stem cells and knock-out and knock-down studies have shown that *BMI1* expression is required for the maintenance of glioma, medulloblastoma, and neuroblastoma cells (Molofsky et al., 2003; Bruggeman et al., 2005; Molofsky et al., 2005; Bruggeman et al., 2007; Cui et al., 2007; Wiederschain et al., 2007; Michael et al., 2008; Abdouh et al., 2009). Our study adressed two important questions: i. Does an increased expression of *Bmi1* in neural stem/progenitor cells lead to the initiation of tumor development (e.g. via promotion of an unlimited self-renewal program) – a concern which needs to be investigated before exploiting BMI1-mediated pathways as a means of expanding normal stem cells and ii. How does BMI1 exert its reported functions – as they can only in part be explained by the repression of *Ink4a-Arf* - and as few target genes have been identified so far (Bracken et al., 2007; Abdouh et al., 2009).

We investigated the consequences of *Bmi1* over-expression in neural stem/progenitor cells from the postnatal murine LVW, and found that elevated BMI1 levels promote the self-renewal and expansion of neurosphere cells *in vitro*. Our results suggest that BMI1 stimulates proliferation and inhibits apoptosis, which - as reported in previous studies - involves the BMI1-mediated repression of the tumor supressors INK4A/ARF and $p21^{CKI}$ (Molofsky et al., 2003; Molofsky et al., 2005; Fasano et al., 2007). As *Ink4a* and *Arf* expression is typically increased in cultured cells, an effect related to "cell culture stress" (Sherr and DePinho, 2000; Lowe and Sherr, 2003), a BMI1-mediated restriction of apoptosis via *Ink4a*/*Arf* might have a stronger impact on cell survival *in vitro* than *in vivo*. This might explain why *Bmi1* over-expression in neural stem/progenitor cells has only little effect on self-renewal and survival *in vivo* compared to a strong effect *in vitro*, as reported by He et al. (2009). However, a recent publication by Yadirgi et al. (2011) found a clear effect of *Bmi1* over-expression on self-renewal and apoptosis of neural stem/progenitor cells *in vivo*, and one might speculate that these observations result from *Ink4*/*Arf*-independent effects of BMI1.

Although *Bmi1* over-expression increased cell proliferation and viability in postnatal neural stem/progenitor cells, this did not lead to tumor development. He et al. did not observe tumor initiation in their mouse model, in which the *Bmi1* transgene was expressed under the control of the Nestin second intron enhancer. The study by Yadirgi et al. (2011), which focused on the *in vivo* over-expression of *Bmi1* in fetal and postnatal neural stem cells, also reported that no tumor initiation could be observed (Yadirgi et al., 2011). Our experiments add further support to these results, as no tumors developed when transplanting a large number of cells over-expressing *Bmi1* by a strong CMV promoter into the brains of recipient mice. Therefore, *Bmi1* might rather be a facilitator of

transforming events induced by other oncogenes. This function of *Bmi1* could be explained by the inhibition of apoptosis (Jacobs et al., 1999a; Abdouh et al., 2009), as well as the maintenance of mitochondrial function and repression of pro-oxidant functions of p53 (Chatoo et al., 2009; Liu et al., 2009), both of which are particularly relevant for tumor cells to counter oncogene-induced apoptosis or senescence, and to prevent the detrimental effects of oxidative stress.

Our gene expression analyses revealed many genes which are repressed in *Bmi1*-over-expressing cells, among which were the hitherto reported direct BMI1 target loci *Cdkn1a* and *Cdkn2a*. In addition, we identified four novel target genes, which are down-regulated in Bmi1-over-expressing cells and are bound by BMI1 in their genomic regions: *Ndn*, *EphA7*, *Rps6ka6*, and *Trp53bp2.*

NDN is an imprinted gene and is down-regulated in several tumor cell lines (e.g. neuroblastoma, glioma (Nakada et al., 2000), melanoma (Hoek et al., 2004) and ovarian cancer (Varma et al., 2005) cell lines). Its protein product has been suggested to act as a tumor suppressor (Chapman and Knowles, 2009), as it interacts with p53 (Taniura et al., 1999) and functions similar to RB via repression of E2F1-mediated transcription (Taniura et al., 1998). Additionally, a regulatory connection between *NDN* and *BMI1* has been proposed: NDN participates in the regulation of *BMI1* via E2F (Kuwako et al., 2004; Chapman et al., 2008) and, *NDN* expression levels have been found up-regulated in a *BMI1* knock-down study (Douglas et al., 2008).

The second BMI1-repressed target gene, which we identified encodes the Ephrin receptor EphA7, which promotes apoptosis of neural progenitors *in vivo* (Depaepe et al., 2005) and induces Ephrin-A2 reverse signaling, thus inhibiting neural progenitor cell proliferation and neurogenesis (Holmberg et al., 2005).

Rps6ka6 encodes RSK4, a protein which confers p53-dependent growth arrest (Berns et al., 2004). *Rps6ka6* is expressed in most cell types (Dummler et al., 2005), but deleted in lung and colon carcinomas (LLeonart et al., 2006). Knock-down of *Rps6ka6* is associated with decreased $p21^{CIP}$ mRNA levels, which results in a bypass of the p53 pathway. Thus RSK4 function contributes to replicative, stress and oncogene-induced senescence (Lopez-Vicente et al., 2009; Lopez-Vicente et al., 2011).

The fourth identified BMI1 target, *Trp53bp2,* codes for the tumor suppressor protein ASPP2 (apoptosis-stimulating protein of p53 2). ASPP2 binds to several transcription factors of the p53 family and enhances their transactivation of pro-apoptotic target genes (Samuels-Lev et al., 2001; Bergamaschi et al., 2004; Vives et al., 2006). Furthermore, ASPP2 has been reported to maintain cell polarity by binding to the polarity complex protein PAR3 in fetal neural progenitors. Knock-out mutations of *Trp53bp2* result in a disruption of the neuroepithelial development and abnormal neural progenitor proliferation (Sottocornola et al., 2010).

Based on the reported functions of the four novel direct BMI1 targets in the regulation of apoptosis and proliferation, their BMI1-mediated repression could contribute to the detected effects of *Bmi1* over-expression in postnatal neural stem/progenitor cells. Future knock-down studies of these four genes in neural stem/progenitor cells should reveal to which extent their individual repression affects the proliferation, self-renewal and survival of these cells.

MATERIALS AND METHODS

Neurosphere (NSP) culture

Lateral ventricle wall tissue was dissected from 4 week-old CD1 mice (Charles River), cells were dissociated with Accutase (PAA) and filtrated (cell strainer 50 µm, BD Biosciences). Single cells were plated in untreated flasks at a density of approximately $1x10^5$ cells/mL and cultured at 37°C and 5% CO_2 in NSP medium, containing DMEM/F12 (1:1) + GlutaMax (Invitrogen), B27 (1x), penicillin (100 units/mL), streptomycin (100 µg/mL, all from Invitrogen), HEPES (10 mM), Partricin (0.5 µg/mL, Biochrom), insulin (20 µg/mL, Sigma-Aldrich), EGF and FGF (both 20 µg/mL, PAN Biotech). For passaging, NSPs were dissociated after 7-9 days with Accutase, cell numbers were determined with a cell counter (CASY technology) and replated at $1x10^5$ cells/mL.

Retroviral transduction

The pCMMP-IRES2-eGFP retroviral vector was kindly provided by Laurent Roybon (modified version of the vector generated by Rogelius et al. (2005). Mouse *Bmi1* cDNA was generated by reverse transcription from total NSP RNA followed by PCR with *Bmi1*-specific primers and inserted into pCMMP-IRES2-eGFP upstream of the IRES site. Two different vector constructs, one with a C-terminal FLAG-tag (pCMMP-Bmi1-FLAG) and one without the tag (pCMMP-Bmi1), were generated (see Supplementary Figure S1).

BD EcoPack2^{TM}-293 cells were used for the production of retroviruses. $6x10^6$ cells were seeded in CellBind flasks (Corning) and kept in medium, containing DMEM (1g/L glucose, Lonza), 10% FBS, 1 mM sodium pyruvate (both from Biochrom), 4 mM glutamine, 100 units/mL penicillin/streptomycin (Invitrogen) and 2.5 µg/mL Amphotericin B (PAA). Approximately 18 h later, this medium was substituted by transfection medium (containing 25 µM chloroquine) and cells were transfected by the calcium phosphate method (Mangasarian et al., 1999). After 16 h, the medium was removed and cells were treated with 15% glycerol in HeBS (25 mM HEPES, 140 mM NaCl, 0.75 mM Na_2HPO_4, all from Sigma-Aldrich) and grown in NSP medium containing 10 mM sodium butyrate (Sigma-Aldrich) for 48 h. Medium was then harvested, filtered through 0.45 µm PVDF membrane filters (Millipore) and viruses were concentrated by centrifugation for 2 h at 20000 x g. Viral pellets were resuspended in 1 mL NSP medium containing 4 µg/mL polybrene (Sigma-Aldrich) and mixed with $3x10^6$ NSP cells. After a concentrated incubation in 1 mL medium for 1 h, cells were resuspended in 15 mL NSP medium and kept for 4 h at 37°C and 5% CO_2. Subsequently, a medium change with fresh NSP medium without polybrene was performed and cells were cultured for one week at 37°C and 5% CO_2 in Ultra Low Attachment flasks (Corning). GFP-positive transductants were purified by flow cytometry (FACS DiVa, BD Biosciences).

In vitro neurosphere assays

To measure self-renewal and proliferation, cells transduced with pCMMP-Bmi1, pCMMP-Bmi1-FLAG and empty vectors were plated in triplicates at clonal density (2000 cells per well, i.e. 1 cell/µL or 1-2 cells/cm^2) in standard 6-well plates (TPP). At each passage (7-10 days after plating), NSPs were counted microscopically. After dissociation with Accutase and resuspension in NSP medium, cell

numbers for each well were determined with a hematocytometer (Neubauer chamber; Roth). This clonal assay was repeated for 8 passages.

Apoptosis and dye retention assay

For the apoptosis assay, early passage pCMMP-Bmi1-FLAG and empty vector control NSP cultures were dissociated with Accutase and washed in PBS. Aliquots of single cell suspensions containing $1x10^5$ cells in 100 µL PBS were stained with AnnexinV-APC and 7AAD according to the manufacturer's instructions (BD Biosciences) for 15 min at RT. Subsequently, 400 µL of AnnexinV binding buffer was added (10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$), cell suspensions were placed on ice and immediately analyzed with a FACS DiVa (BD Biosciences).

For the dye retention assay, passage six pCMMP-Bmi1-FLAG and empty vector NSP cultures were dissociated into single cells with Accutase and washed in 1% BSA (in PBS). Subsequently, cells were centrifuged, resuspended in Diluent C and stained with PKH26 in ethanol (1 µM staining solution; both from Sigma-Aldrich) for 5 min. The staining reaction was terminated by addition of 500 µL 1% BSA (in PBS) and followed by two washing steps. Finally, cells were seeded under clonal conditions in untreated flasks. After six days in culture, PKH26 intensities of dissociated NSP cells were analyzed by flow cytometry (FACS DiVa, BD Biosciences). Unstained cells and cells stained directly before analysis served as negative and positive control respectively.

Flow cytometric data were analyzed and plotted with FlowJo (TreeStar). One-way ANOVA with Bonferroni's multiple comparison post-hoc test was used for the statistical analyses of theses assays (PRISM, GraphPad).

Microarray analysis

Total RNA was extracted according to the manufacturer's instructions using the AllPrep DNA/RNA kit (Qiagen). Concentration, purity and integrity of the RNA were assessed with the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip® (Agilent Technologies). 300 ng of total RNA was used for the first strand cDNA synthesis. Sample preparation and hybridization to Affymetrix Mouse Gene ST 1.0 arrays, washing, and scanning were performed according to the Affymetrix standard protocol (GeneChip Whole Transcript (WT) Sense Target Labeling Assay User manual, P/N 701880 Rev.5). Microarray data was normalized by the Robust Multi-array Average (RMA) method using the Expression Console software. MaxDView software was used to further analyze the normalized data (http://www.bioinf.manchester.ac.uk/microarray/maxd/maxdView/). A t-test was applied to analyze and compare *Bmi1*-over-expressing and empty vector control samples (n=3 of each). Differential expression of genes was considered relevant, when a p2value below 0.001 and a mean log difference of at least 1 was found.

Intracranial transplantation

pCMMP-Bmi1, pCMMP-Bmi1-FLAG and empty vector NSP cultures from an early passage as well as GL261 murine malignant glioma cells (which were kept in DMEM (4.5g/L glucose, Lonza), 10% FBS, 2 mM glutamine, 100 units/mL penicillin/streptomycin (Invitrogen) and 2.5 µg/mL Amphotericin B (PAA)) were dissociated to single cell suspensions and $3x10^5$ cells in 3 µL medium were transplanted into female 4 week-old CD1 and immunocompromised nude mice. Mice were anesthetized by intraperitoneal injections of a ketamine/xylazine solution (2mg ketamine, 0.2mg xylazine per 30 g mouse). Cells were injected 2.5 mm lateral, 1.5 mm anterior of the bregma and 3 mm deep into the right frontal brain lobe with 10 µL Hamilton syringes (Hamilton Bonaduz AG). Transplanted animals were monitored for 6 months. All procedures were performed with consent from the ethical committee at Lund University.

Gene Set Enrichment Analysis (GSEA)

GSEA was applied as described (Subramanian et al., 2005). To rank the genes according to the two phenotypes (comparing empty vector controls with *Bmi1* over-expressing cells), the difference of the normalized intensity (i.e. logarithmic values) was used as a metric. Phenotype permutations (1000) were chosen, as only triplicate data sets were available per condition. As we investigated the enrichment of a relatively low number of gene sets, we applied a stringent statistical cut off, considering only gene sets with a family wise error rate (FWER) < 0.01 as relevant. Detailed information about gene sets can be found in Supplementary File S1.

Chromatin Immunoprecipitation (ChIP)

NSP cells transduced with pCMMP-Bmi1-FLAG and empty vectors from early passages were cultured as described above for 7 days. To cross-link DNA and proteins, formaldehyde (Merck) was added to the culture medium at a final concentration of 1%. Cross-linking was allowed to proceed for 10 min at room temperature and stopped by addition of glycine (Scharlau) at a final concentration of 12.5 mM, followed by an additional incubation of 5 min. Cells were washed with ice cold PBS (Lonza) containing 2 mM $MgCl_2$ (OMNI Life Sciences) and resuspended in 1 mL lysis buffer (Dahl and Collas, 2008) with a protease inhibitor cocktail (Roche) and 1 mM PMSF (Roche). After an incubation of 20 min at room temperature, the cell suspension was transferred to 15 mL sample tubes (Greiner BioONE) with 200 mg PBS-washed glass beads (Sigma-Aldrich) for disruption. The ice-cooled cells were sonicated four times for 30 sec with 1 min breaks in between at power level 4 and 50% duty cycle with a Branson 450 probe, yielding DNA fragments with a bulk size of 100-400 bp. The debris-cleared lysate was aliquoted and stored at -80°C.

Slurry of magnetic Dynabeads (Invitrogen) was mixed 1:2 with PBS-based blocking buffer (Lonza) containing 1 mg/mL Herring Sperm DNA (Promega) and 1 mg/mL BSA (molecular grade, Sigma-Aldrich) and incubated over night at 4°C on a rotator. For each reaction, beads contained in 100 µL of the slurry-blocking buffer mix were separated with a DynaMagTM magnet (Invitrogen) and incubated for 20 min at room temperature with either monoclonal anti-FLAG-antibody M2 (5 µg, Sigma-Aldrich) or IgG1-antibody (5 µg, Millipore, negative control) diluted in 200 µL binding & washing buffer

(Invitrogen). For each immunoprecipitation, 100 µL cell lysate were diluted with 900 µL RIPA buffer (Dahl and Collas, 2008) containing PMSF and protease inhibitor and incubated with the prepared beads for 2 h at room temperature on a rotator. Beads were washed 3 times with washing buffer (Invitrogen) and then transferred to clean tubes. Cross-linking was reversed by resuspension of the beads in 250 µL digestion buffer containing 10 mM Tris (Scharlau), 10 mM EDTA (Merck) and 1% SDS (Calbiochem), addition of proteinase K (Fermentas) to a concentration of 50 µg/mL, and incubation for 2 h at 68°C on a shaker (800 rpm). 10 µL of raw lysate obtained after sonication were diluted in 240 µL digestion buffer and treated equally to the ChIP samples, serving as input control. Finally, the material was phenol-chloroform extracted and ethanol precipitated. DNA was resuspended in 50 µL of nuclease-free water (Ambion) and 2 µL were used as template for PCR. Primers used to amplify mouse genomic sequences at BMI1 target loci are listed in Supplementary Table S2.

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FIGURE LEGENDS

Figure 1. *Bmi1* over-expression increases proliferation and self-renewal of adult NSP cells *in vitro* but does not transform NSP cells into tumor initiating cells.

(A) Frequency of neurosphere initiation of empty vector, pCMMP-Bmi1 and pCMMP-Bmi1-FLAG transduced cells assessed 7 days post plating for 8 passages (n=3). (B) Total cell numbers measured at 7-9 days post plating over 8 passages (n=3). Error bars in (A) and (B) represent standard deviations (C) Box plots representing neurosphere diameters determined for empty vector, pCMMP-Bmi1 and pCMMP-Bmi1-FLAG transduced NSP cells at passage 2 (50 spheres were investigated in 3 independent cultures). Error bars represent the 10-90th percentiles. (D) Fluorescent micrographs of empty vector and *Bmi1*-FLAG transduced neurospheres. (E) Tumor formation in mice intracranially transplanted with NSP cells transduced with empty vector, pCMMP-Bmi1-FLAG and pCMMP-Bmi1 as well as GL261 control cells (n.d. = not determined). The number of developed tumors / number of transplanted animals is shown..

Figure 2. Over-expression of *Bmi1* stimulates *in vitro* proliferation of NSP cells and has a survival-enhancing effect.

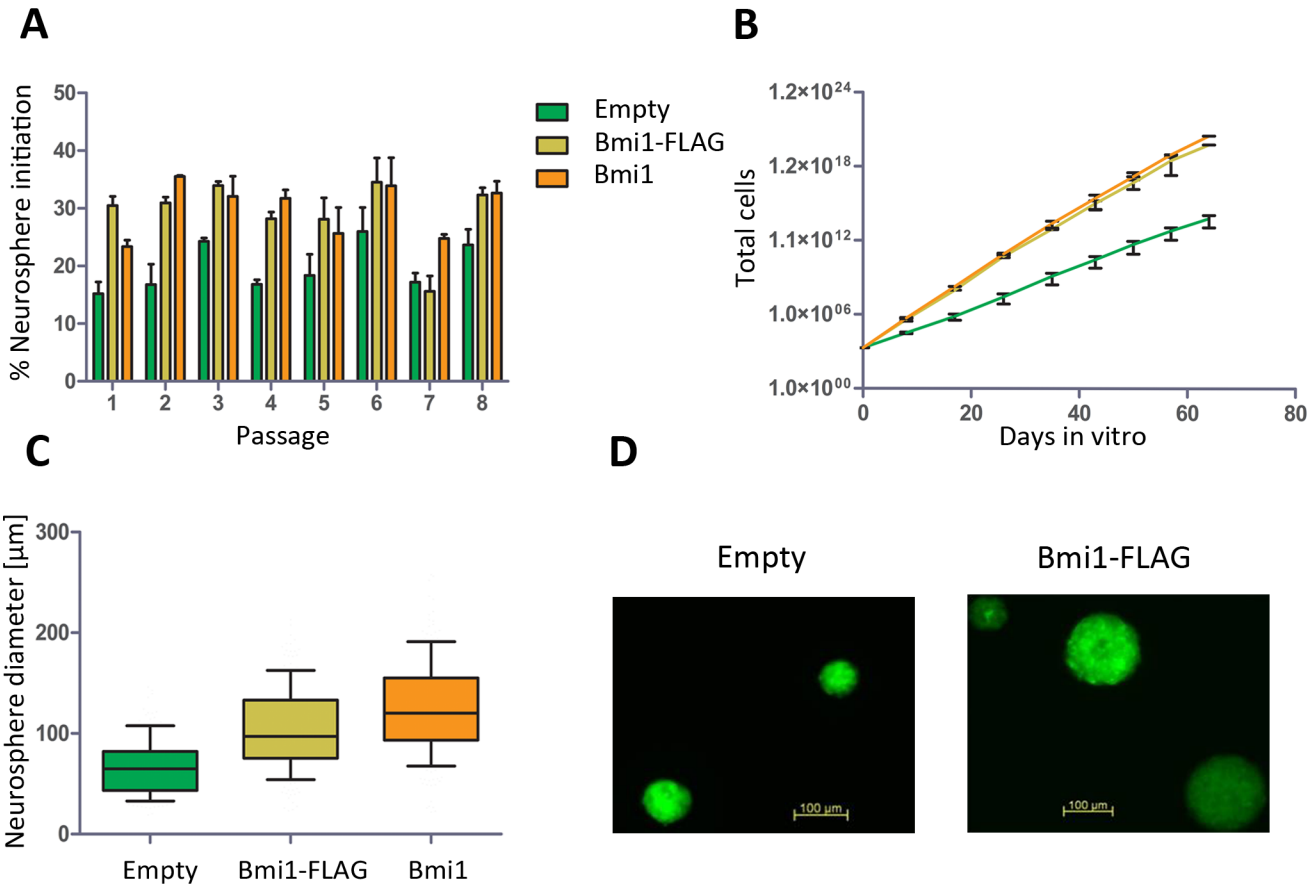
(A) Apoptosis assay of *Bmi1*-FLAG, *Bmi1* or empty vector (Empty) transduced NSP cells from passage 3 stained with AnnexinV and 7AAD to determine the amount of dead ($AnnexinV^+/7AAD^+$), living ($AnnexinV^-/7AAD^-$) and early apoptotic ($AnnexinV^+/7AAD^-$) cells. (B) Mean fluorescence intensity (log values) of NSP cells transduced with empty vector, *Bmi1*-Flag, or *Bmi1* labeled with PKH26 and cultivated for six days before FACS analysis. Unstained cells and cells stained prior to flow cytometry analysis served as negative and positive controls, respectively. All data was tested for statistical significance applying the one-way ANOVA and a Bonferroni Multiple Comparison post-hoc test. Error bars represent standard deviations; n=3; **: $p<0.01$, ***: $p<0.001$; ns = not significant.

Figure 3. *Bmi1* over-expression effects on the gene expression profile of neural stem/progenitor cells

(A) Plots of the two gene sets, which were found to be enriched upon over-expression of *Bmi1* according to GSEA (FWER<0.01) (see also Supplementary Table S1). Vertical black lines, representing components of the gene sets, are positioned along the ranked differential gene expression values of the experiment (genes up-regulated upon *Bmi1* are shown on the far right, genes down-regulated on the far left). The enrichment of the gene set components at the right side reflects their over-representation among BMI1-up-regulated genes. (B) Selected down-regulated genes in *Bmi1*-over-expressing cells which belong to different functional categories.

Figure 4. Identification of direct BMI1 targets.

Representative agarose gel electrophoresis images of PCR-amplified *Ink4a, EphA7*, *Ndn, Rps6ka6* and *Trp53bp2* genomic regions using material from ChIP samples and input controls as template (n=3). ChIP was performed with empty vector (empty) and pCMMP-Bmi1-FLAG transduced NSP cells, applying the anti-FLAG antibody M2. A matched IgG1 isotype antibody was used as negative control and post-sonication cell lysate served as input control.

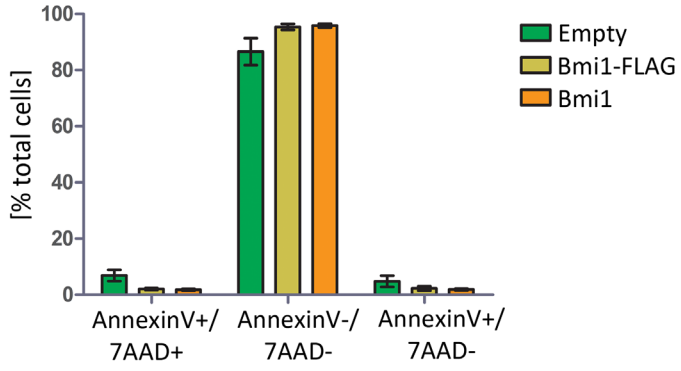


E

	CD1 mice	Nude mice
Empty vector controls	0/6	0/4
Bmi1	0/3	0/2
Bmi-FLAG	0/6	0/4
GL261	n.d.	3/3

FIGURE 1

A


100
80
60
40
20
0
[% total cells]
AnnexinV+/ 7AAD+
AnnexinV-/ 7AAD-
AnnexinV+/ 7AAD-
Empty
Bmi1-FLAG
Bmi1

B

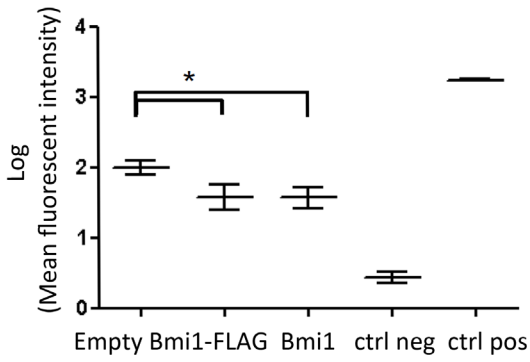
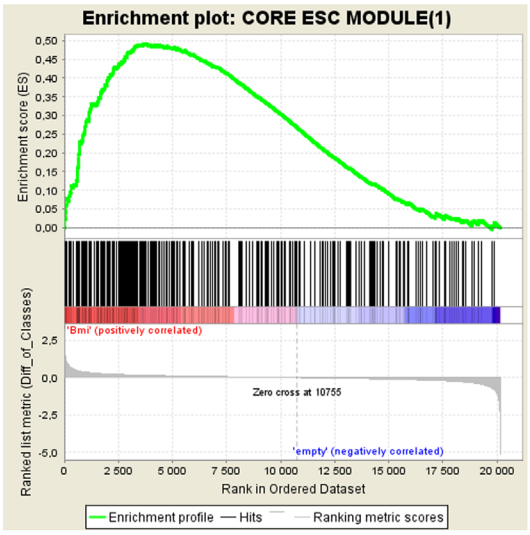
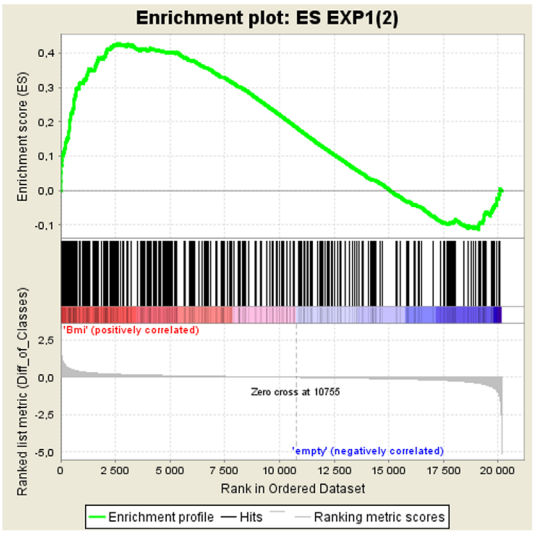

Log (Mean fluorescent intensity)
4
3
2
1
0
*
Empty
Bmi1-FLAG
Bmi1
ctrl neg
ctrl pos

FIGURE 2

A





B

Gene	Fold change
Imprinting	
Tssc4	2.2
Pygm	2.8
Snord15	**
Snord16	5.1
Snurf	3.0
Ndn	3.4
Sgce	2.1
Peg10	3.6
Apoptosis	
Slit2	3.4
EphA7	14.2
Tumor suppression	
Frk	3.7
Igfbp4	3.1
Unc5b	2.3
Tmeff2	3.5
Cdkn1a	2.1
Cdkn2a	2.1
Oxidative stress	
Dusp4	2.0
Senescence	
Gas7	2.6

FIGURE 3

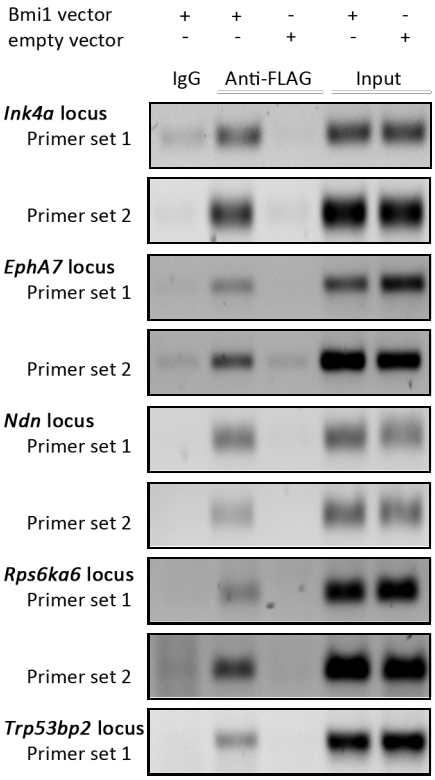

Bmi1 vector + + - + -
empty vector - - + - +
IgG
Anti-FLAG
Input
Ink4a locus
Primer set 1
Primer set 2
EphA7 locus
Primer set 1
Primer set 2
Ndn locus
Primer set 1
Primer set 2
Rps6ka6 locus
Primer set 1
Primer set 2
Trp53bp2 locus
Primer set 1

FIGURE 4

Supplementary information

Identification of novel BMI1 targets in neural stem/progenitor cells

Falk Hertwig[1,2,3], Sebastian Braun[1,3], Ulrike A. Nuber[1]

[1] Lund Strategic Center for Stem Cell Biology, Lund University, 22184 Lund, Sweden
[2] Section Biology, Chemistry and Pharmacy, Freie Universität Berlin, 14195 Berlin, Germany
[3] These authors contributed equally

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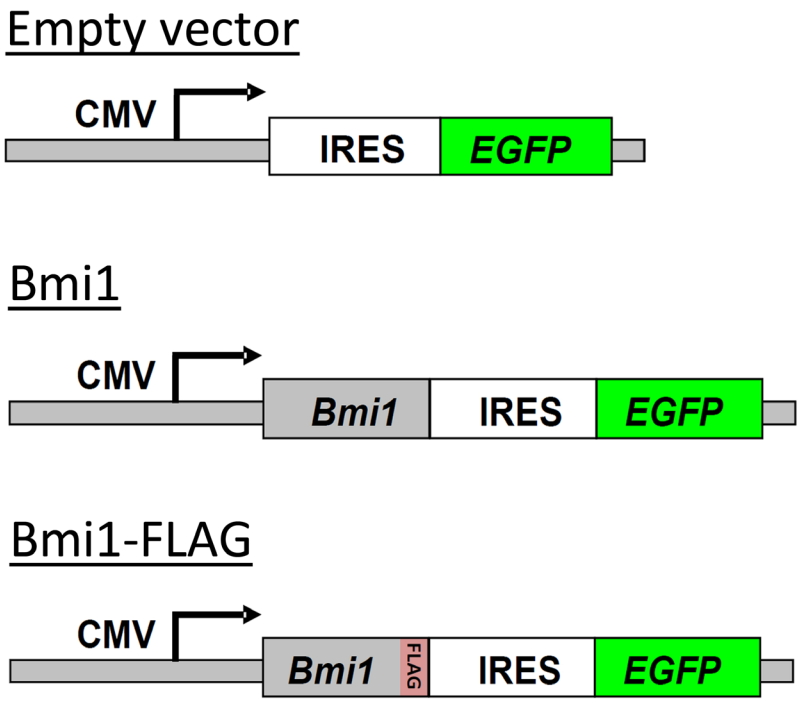
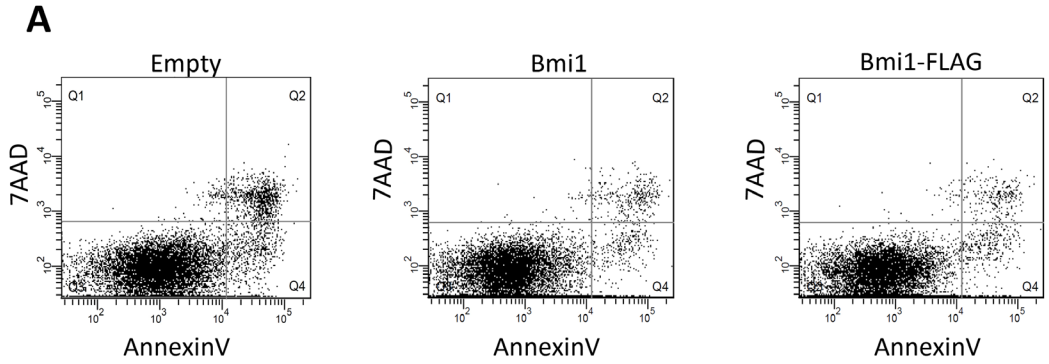
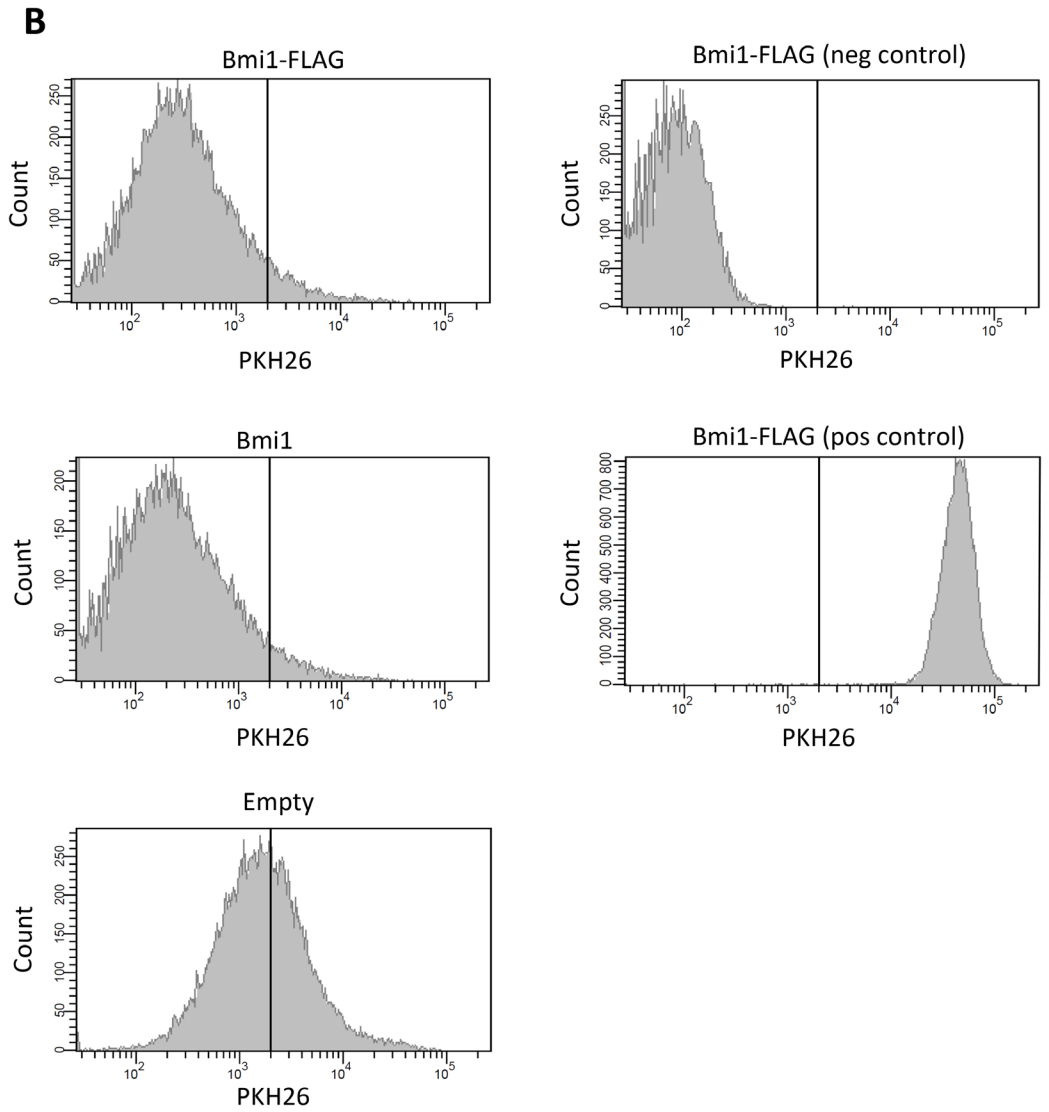
Supplementary Figure S1 – Retroviral constructs used in this study

Schematic of the empty vector, pCMMP-Bmi1 and pCMMP-Bmi1-FLAG expressing retroviral constructs. The bicistronic vector construct expresses *Bmi1* under a CMV promoter along with *EGFP* and contains an internal ribosomal entry site (IRES) site.



Supplementary Figure S2 – Flow cytometric apoptosis and proliferation assay
(A) Representative FACS dot plots displaying empty vector (Empty) or pCMMP-Bmi1-FLAG transduced neurosphere cells (passage 3) stained with AnnexinV-APC and 7AAD. Q2 (AnnexinV$^+$/7AAD$^+$) = dead cell fraction, Q3 (AnnexinV$^-$/7AAD$^-$) = living cell fraction, Q4 (AnnexinV$^+$/7AAD$^-$) = apoptotic cell fraction. (B) Representative FACS histograms displaying PKH26 fluorescence intensity of NSP cells transduced with empty vector (empty), pCMMP-Bmi1 or pCMMP-Bmi1-FLAG. Cells were labeled with PKH26 and cultivated for six days prior to FACS analysis. Unstained cells and cells stained prior to FACS analysis served as negative and positive control, respectively.





Supplementary Table S1 – Gene Set Enrichment Analyses (GSEA)[1] of the *Bmi1* over-expression profile

Gene set (GS)	GS size	NES	FWER p-value
Enriched in *Bmi1* over-expressing cells			
CORE ESC MODULE[a]	312	2.10	0.000
ES EXP1[d]	334	1.85	0.001
MEDULLOBLASTOMA[c]	77	1.66	0.018
MALIGNANT GLIOMA[c]	73	1.53	0.080
H3K27 BOUND[d]	938	1.53	0.089
PRC MODULE[b]	511	1.52	0.093
MOUSE ESC-LIKE MODULE[a]	1214	1.49	0.108
HUMAN ESC-LIKE MODULE[a]	1090	1.48	0.118
PRC2 TARGETS[d]	550	1.46	0.140
SUZ12 TARGETS[d]	871	1.44	0.155
EED TARGETS[d]	872	1.43	0.172
OCT4 TARGETS[d]	255	1.22	0.523
MYC TARGETS1[d]	211	1.11	0.837
CORE MODULE[b]	102	1.11	0.841
MYC MODULE[b]	461	0.96	1.000
MYC TARGETS2[d]	685	0.91	1.000
ES EXP2[d]	36	0.85	1.000
Enriched in empty vector control cells			
NOS TFS[d]	35	1.72	0.016
MOUSE ADULT TISSUE STEM MODULE[a]	694	1.63	0.043
AT/RT[c]	91	1.49	0.123
NANOG TARGETS[d]	821	1.26	0.578
NORMAL CEREBELLUM[c]	86	1.23	0.647
SOX2 TARGETS[d]	615	1.21	0.715
PNET[c]	64	1.19	0.789
NOS TARGETS[d]	159	1.09	0.976

[a] Wong et al.- Cell Stem Cell 2008; [b] Kim et al. - Cell 2010; [c] Pomeroy et al. - Nature 2002; [d] Ben-Porath et al. - Nature Genetics 2008

[1] GSEA to identify a correlation between gene expression signatures of human brain tumors and mouse stem cells and the *Bmi1* over-expression profile. Relevant enriched gene sets (FWER < 0.01) are highlighted. Detailed information about gene sets and their components can be found in Supplementary File S1. NES = absolute normalized enrichment score

Supplementary Table S2 – Primer pairs used for chromatin immunoprecipitation (ChIP) assays

	Forward primer	**Reverse primer**
***Ink4a* locus**[a]		
Primer set 1	5'-CAG ATT GCC CTC CGA TGA CTT C-3'	5'-CCA AGC TGA GAT CCC AAC AAC C-3'
Primer set 2	5'-GGT TCG ACT CTA GGG TTG TTG G-3'	5'-TGG ACC CGC ACA GCA AAG AAG T-3'
***EphA7* locus**		
Primer set 1	5'-GTT GTT GTG AAC TCA GGC AAA C-3'	5'-CGG ACA GTG AAA CTG AGC TAT G-3'
Primer set 2	5'-TGC AGT AGC TAA AAG CCA AGT G-3'	5'-TTG CTC CAC ACT CCA ATA ATA TC-3'
***Ndn* locus**		
Primer set 1	5'-AAC TCA TCA TCA TCA TAA GGT ACA GC-3'	5'-GAT TTC CTG GTC TCC TGT ATG G-3'
Primer set 2	5'-GCC TAG TGG TAC CCT CCC TTA G-3'	5'-CGC TCA GGT CCT TAC TTT GTT C-3'
***Rps6ka6* locus**		
Primer set 1	5'-GTG TTG GGT GCA CTT GTT TG-3''	5'-AGC GAG AGG CAG CAC ATA AT-3'
Primer set 2	5'-CCA GGC AGC TTC TTC TCG-3'	5'-CGG GCT GCA GAC TTA CAA C-3'
***Trp53bp2* locus**		
Primer set 1	5'-CCT AAA CCC CTC ATC AAC TCA C-3'	5'-AAA AAT TTA GGA ACA CCC TCC AC-3'

[a] Primers at the *Ink4a* promoter region were adapted from Matheu et al., 2005