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Vorstand: Prof. Dr. med. Dr. h.c. H. A. Kretzschmar

The role of β1-Integrin in the cerebellar development and the formation of medulloblastoma

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Proper cerebellar development requires expression of β1-Integrin in Bergmann glia, but not in granule neurons

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Contents

1. Introduction

1.1. Adhesion receptor β1-Integrin

β1-Integrin, a subunit of the Integrin receptor family, plays a key role as a regulator of both development biology and the formation of cancer (Brakebusch et al. 1997; Fassler and Meyer 1995; Lahlou and Müller 2011; Morello et al. 2011; Stephens et al. 1995; White et al. 2004).

In general an Integrin consists of one α and one β subunit (Hynes 2002). 18 different α subunits and 8 different β subunits, which form 24 Integrin heterodimers, have been found in organisms. Remarkably, 12 of these Integrin heterodimers contain the β1-Integrin subunit (Hynes 2002).This fact emphasizes its great importance additionally to its ubiquitous expression.

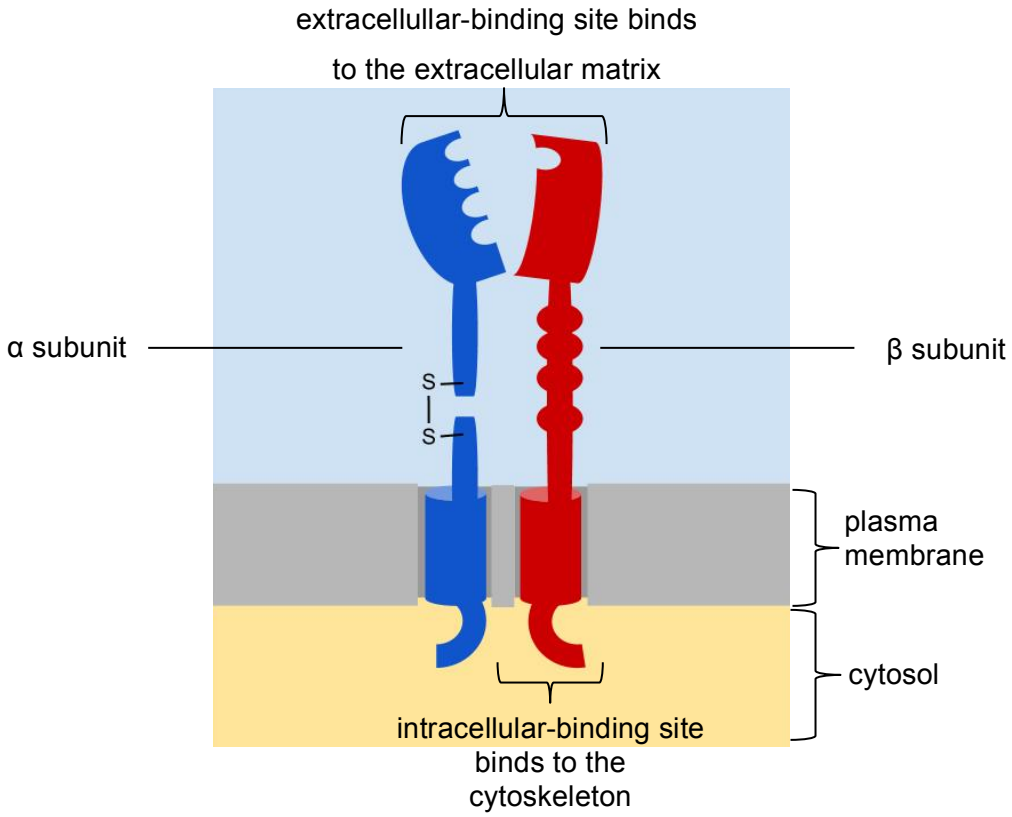

Figure 1 (Modified from (Weinberg 2007)) Schematic structure of Integrins. The α subunit (blue) and the β subunit (red) form a heterodimer of Integrin, a transmembrane adhesion cell receptor, spanning the cell membrane once. The outer domain of the Integrin attaches the cell to components of extracellular matrix (ECM) or adjacent cells. The intracellular domain of Integrin mediates the connection to the cytoskeleton by linking proteins.

A special feature of Integrins is their ability to signal bidirectionally (Hynes 2002) without being enzymatically active (Liu et al. 2000). Integrins are involved in

proliferation, apoptosis, migration and differentiation processes of cells (Hu and Luo 2012; Hynes 2002).

These are essential steps of developmental biology and as formerly shown, β1-Integrin is crucial for the early development of the mouse embryo. Mice with a homozygous, constitutive knockout of β1-Integrin die around E6.5 (embryonic age day 6.5) because of degenerative growth of the inner cell mass of the blastocyst (Brakebusch et al. 1997; Fassler and Meyer 1995; Stephens et al. 1995).

In further development of the organism β1-Integrin is critical especially in the formation of mammary gland (Klinowska et al. 1999; Li et al. 2005; Liu et al. 2000; Naylor et al. 2005) and the development of the skeletal muscle (Brakebusch et al. 1997; Burkin et al. 2001; Schwander et al. 2003).

The fact that β1-Integrin is also important for the development of the cerebral and cerebellar cortex is of special interest for this research project (Belvindrah et al. 2007; Blaess et al. 2004; Dulabon et al. 2000; Graus-Porta et al. 2001; Rice and Curran 2001).

Breast cancer is a prime example that β1-Integrin is not only decisive in developmental processes but also in cancer formation (Lahlou and Müller 2011). In a genetically modified mouse model, White et al. showed that β1-Integrin is responsible for the induction and maintenance of breast cancer (White et al. 2004). Moreover, increased β1-Integrin expression is correlated with a poor survival of human breast cancer patients. According to this, it may serve as a prognostic factor for these patients (Yao et al. 2007).

In several studies it was shown that β1-Integrin has also notable effects on tumor recurrence, survival, progression and metastasis and is suggested to be a target for cancer therapies (Barkan and Chambers 2011; Cordes and Park 2007; Morello et al. 2011; Park et al. 2006).

1.2. Cerebellar Development

The adult cerebellum is organized in a well-defined, cortical laminar structure with only three layers (Palay and Chan-Palay 1974)(Figure 2).

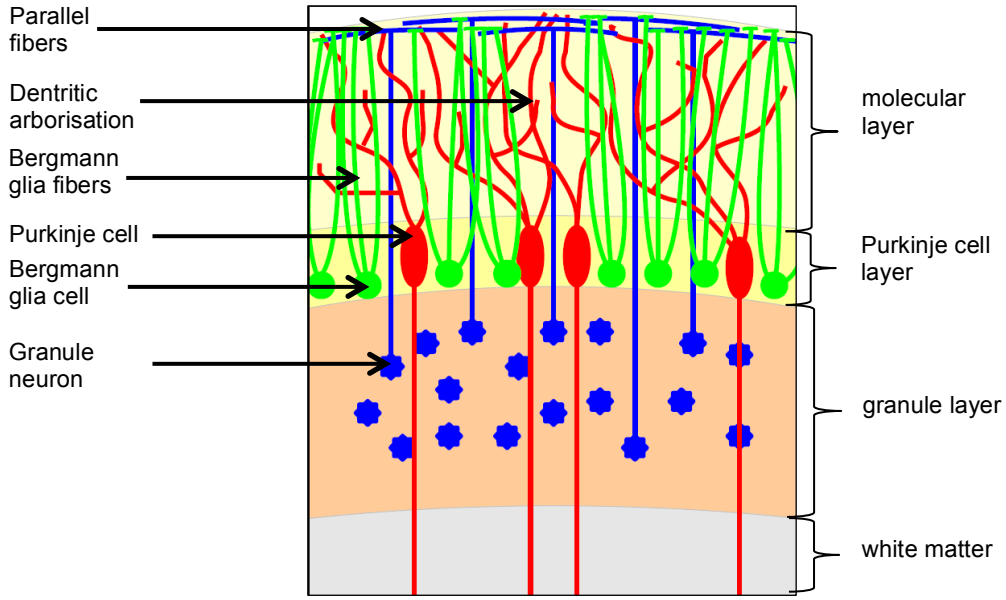

Figure 2 (modified from (Hatten 1999; Tanaka et al. 2008)) Schematic and simplified cross section of the adult cerebellar cortex consisting of three layers with the relevant cell types for this study. The granule layer contains the cerebellar granule neurons (blue), the Purkinje cell layer contains the Purkinje cells (red) and between them are the Bergmann glia cells located (green). The molecular layer contains the T-shaped parallel fibers of granule neurons, the dendritic arborisation of Purkinje cells and the Bergmann glia fibers.

The cells of the CNS can be broadly divided into glial cells and neuronal cells, both differentiate from multipotential neural progenitor cells (Edlund and Jessell 1999). This study about cerebellar development focuses especially on cerebellar granule neurons and Bergmann glia.

The cerebellar granule neuron is by far the most common cell type in the adult cerebellum and forms the innermost granule layer. The following Purkinje cell layer is a monolayer of fan-shaped Purkinje neurons with dentritic arborisation (Palay and Chan-Palay 1974) (Figure 2). The cell bodies of the Bergmann glia lie in the region of the cell bodies of Purkinje cells and their fibers pass the outermost molecular layer to the pial surface (Grosche et al. 2002; Welsch 2006). In addition to the Bergmann glial fibers also the processes of the cerebellar granule neurons, the parallel fibers, and the dentric arborisation of the Purkinje cells extend into the molecular layer (Figure 2).

At the first glance the histological structure of the cerebellar cortex seems quite simple, even more notable is that the function of the cerebellum is more complex than previously assumed. In addition to the well-known motor coordination and control functions of the human cerebellum, its contribution in higher cognitive functions is also suggested by observation of clinical cases and fMRI studies (Fiez 1996; Ramnani 2006).

The cerebellum develops in highly organized processes of proliferation, differentiation, migration and forming circuitry of cells (Altman J 1997). The fourth ventricle is located dorsal of the developing embryo and forms a rhomboid-shaped gap in the neural tube. The fourth ventricle is bordered on its outer edge by the rhombic lip. Rostral from the fourth ventricle the cerebellar anlage is generated. Purkinje cells and inhibitory interneurons originate from the ventricular zone above the fourth ventricle. Then the postmitotic Purkinje cell precursors migrate along the emerging radial glial fibers into the interior of the developing cerebellar anlage and arrange in a monolayer (Misson et al. 1988). Meanwhile the cerebellar granule neuron precursors (CGNPs) arise from the upper rhombic lip proliferate there and move rostral to cover the cerebellar anlage in order to form there the external granule layer (EGL) (reviewed in (Hatten 1999; Hatten and Heintz 1995; Ten Donkelaar and Lammens 2009)). There the CGNPs proliferate enormously, approximately until the second week postnatal (Hatten and Heintz 1995; Miale and Sidman 1961; Roussel and Hatten 2011). During this phase, the Sonic hedgehog (Shh) protein, produced by Purkinje cells, is the major mitogen for CGNPs. If the EGL has reached its maximum thickness of about 8 cells, during further proliferation of the CGNPs the thickness of the EGL does not change, rather the horizontal extent of the EGL increases (Fujita 1967; Fujita et al. 1966). Then the EGL begins to decrease because postmitotic CGNPs differentiate and migrate along the Bergmann glial fibers below the Purkinje cell layer and form there the very compact internal granule layer (IGL). While they are migrating, the granule cells leave their extending axon, the T-shaped parallel fibers, behind in the molecular layer (Gao and Hatten 1993; Gao and Hatten 1994; Hatten and Heintz 1995). According to the observation that CGNPs adhere to and migrate along the Bergmann glial fibers on the way to their final position in the internal granule layer (IGL), it is supposed that adhesion receptors like β1-Integrin may be involved in these processes (Fishman and Hatten 1993; Hatten and Heintz 1995).

In the terminal stage of cerebellar granule neuron differentiation, the cerebellar circuit network establishes and cerebellar granule neurons connect with mossy fibers and the dentritic arborisation of Purkinje cells (Hatten and Heintz 1995).

The knowledge of the development of the cerebellar cortex is obtained mainly by investigation on mutant mice, the prototype for this studies is the reeler mouse (Hatten and Heintz 1995; Rice and Curran 1999). The reeler mouse is lacking reelin, a product of the *reeler* gene and mostly secreted by Cajal Retzius cells in the marginal zone. It is supposed that reelin modulates the neuronal-radial glial interplay and it was shown that reelin inhibits the neuronal migration. The result of the disruption of the *reeler* gene is an abnormal development of the cerebellum and cerebrum. The cerebellum, with a perturbated lamination, is reduced in size and the different cell layers of the cerebrum seem to be inverted. *In vitro* studies of Dulabon et al. connote the binding of Reelin to β1-Integrin and *In Vivo* experiments indicate that α3β1-Integrin is responsible for the proper function of reelin (Dulabon et al. 2000; Rice and Curran 1999).
Furthermore, in a genetically modified mouse model lacking β1-Integrin in neural progenitors were observed laminar disorganization of cerebellar granule neurons and the fusion of folia in the cerebellum and of hemispheres of the cerebrum. These abnormalities were suggested to be caused by disorders in the marginal zone (Graus-Porta et al. 2001). In the same mouse model a decreased size of the cerebellum was observed, Blaess et al. supposed that β1-Integrin may be critical for the proliferation of CGNPs (Blaess et al. 2004). In a further study Belvindrah et al. showed that β1-Integrin in radial glia is required for the proper lamination of the cerebral cortex (Belvindrah et al. 2007) and reactive gliosis in the forebrain was observed in a mouse model with a loss of β1-Integrin in glia (Robel et al. 2009). However, the specific role of β1-Integrin in glia and CGNPs in the cerebellar development is unknown.

1.3 Medulloblastoma

Medulloblastomas are a biological and clinical heterogeneous group of embryonal tumors arising in the cerebellum (Roussel and Hatten 2011) or brainstem (Gibson et al. 2010; Grammel et al. 2012). Besides leukemia, medulloblastoma is one of the most common malignancies in childhood.
A good knowledge of the cerebellar development is required in order to understand the formation of medulloblastoma. Mutations and deregulations of genes and pathways that are crucial for a correct cerebellar development can lead to its formation (Gibson et al. 2010; Roussel and Hatten 2011). Well investigated pathways of cerebellar development are the wingless pathway (WNT-pathway) and sonic hedgehog pathway (SHH-pathway), which show abnormal activation in medulloblastomas. Recently, these pathways gained on significance in molecular identification of the four distinct subgroups of medulloblastomas by the application of whole-genome and other

sequencing techniques (Kool et al. 2012; Northcott et al. 2011; Taylor et al. 2012). The WNT and SHH tumors represent two of these four genetic different subgroups. The other 2 subgroups, called the Group 3 and the Group 4, are molecularly not that well defined (Kool et al. 2012; Taylor et al. 2012)(Figure 3).

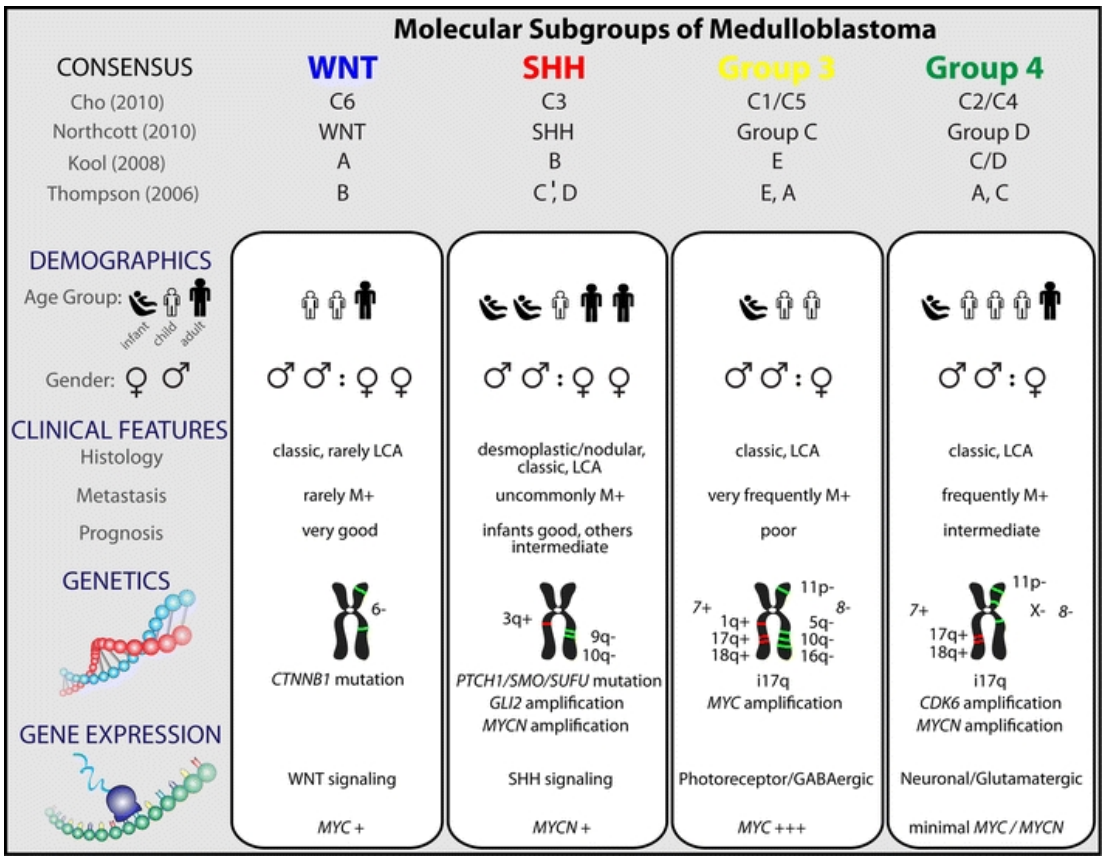

Figure 3 (Taylor et al. 2012) The current consensus of molecular subgroups of medulloblastoma. Four distinct molecular subgroups were identified through transcriptional profiling studies from several research groups around the world. This overview summarizes the clinical, demographic and genetic data of the four subtypes. LCA= large cell/anaplastic tumors.

However, medulloblastoma are still diagnosed according to the criteria of the 2007 WHO classification of brain tumors (Louis et al. 2007) in clinical practice. Based on histological und immunhistochemical methods there are classic, desmoplastic, extensive nodular and large cell medulloblastomas. All subtypes of medulloblastoma are classified as tumors of highest malignancy, WHO grade IV (Louis et al. 2007).

Besides the progress with respect to the molecular classification of the medulloblastoma, the cell of origin is another important topic of recent medulloblastoma research. In mice, the SHH-associated medulloblastomas may arise from CGNPs in the upper rhombic lip (Schüller et al. 2008) or cochlear granule neuron precursors (Grammel et al. 2012) and the Wnt-associated tumors may arise from pontine grey

neuronal progenitors within the lower rhombic lip and the dorsal brain stem (Gibson et al. 2010).

Assuming that a medulloblastoma originates from immature neuronal cells, also the statistics of it reflects the fact that this is a typical embryonal tumor. The percentage of embryonal brain tumors including medulloblastomas compared to all primary brain tumors decreases from 15.1% in childhood (Ages 0-14) to 4.3% in adolescent (Ages 15-19) to 1.9% in adults (Ages 20-34) (CBTRUS 2012). 50% of all medulloblastomas occur in children under 5 years (Kaatsch et al. 2001). Furthermore, medulloblastomas have a strong tendency for dissemination through the cerebrospinal fluid in the leptomeningeal space (Gajjar et al. 2006; Wu et al. 2012). Therefore, the age of the patient at diagnosis, the dissemination of the primary tumor and the extent of residual disease after primary resection are important to make a statement about the prognosis and to determine which therapy is applied. Currently, this risk stratification and the subsequent therapies are more and more adapted to the molecular-based classification and the results of recent studies. Formerly, children under 3 years were considered as high risk patients with poor prognosis. In recent studies it was shown, that 65% of medulloblastomas in infants under 3 years of age are tumors of the SHH subgroup with predominantly desmoplastic histology (Northcott et al. 2011; Ramaswamy et al. 2011). Infants with a medullblastoma of this subgroup have good prognosis with postoperative chemotherapy alone (Ramaswamy et al. 2011; Rutkowski et al. 2005; von Bueren et al. 2011). In contrast young children with a medulloblastoma of Group 3 have a very poor prognosis and respond poorly to current therapies (Northcott et al. 2011; Ramaswamy et al. 2011).

In the standard therapy of medulloblastoma the aim of the surgical therapy is to resect the medulloblastoma totally, and in order to reduce the probability of a possible metastasis, the whole craniospinal axis is irradiated. In addition, a chemotherapy is performed (Roussel and Hatten 2011). The therapy can be adapted according to the risk of the patient. Despite enormous advances in diagnostics and therapies, the 5-year overall survival rate is still ~70% and surviving patients suffer from irreversible neurological and cognitive problems caused by the treatment. Therefore, it is important to improve therapies and develop more targeted therapies and molecular markers for diagnostics of medulloblastoma patients. One major advance in targeted therapies referring medulloblastomas was the discovery of the hedgehog-pathway inhibitor, called Vismodigib or GDC-0049, which is already included in a clinical study (Roussel and Robinson 2011; Rudin et al. 2009).

However, additional molecular targets for therapy and diagnostic markers are required. The role of β1-Integrin in medulloblastoma is unknown, but it displays an interesting candidate to fulfill this functions. Therefore the supporting facts for this assumption are summarized:

A critical role of β1-Integrin was suggested for the development of the cerebrum and cerebellum and for the proliferation of CGNPs (Blaess et al. 2004; Graus-Porta et al. 2001), which may give rise to medulloblastoma (Schüller et al. 2008).

Apart from its role in developmental biology, β1-Integrin has a crucial role in tumorgenesis, what has been demonstrated most clearly through breast cancer research (Lahlou and Müller 2011; White et al. 2004; Yao et al. 2007).

1.4. Aim of this study

The first aim of this study is to specify the role of β1 Integrin in CGNPs and glial cells during cerebellar development.

To clarify this, conditional knockout mouse models were used, where β1-Integrin was selectively inactivated once in CGNPs and once in glia cells.

The second aim of this study is to investigate the role of β1-Integrin in the formation of medulloblastoma and if it could serve as a prognostic marker for medulloblastoma patients. Therefore, another conditional knockout mouse model was used to generate a Shh-associated medulloblastoma and delete conditionally β1-Integrin from these tumors. Since the Shh-associated medulloblastomas in this mouse model are derived from CGNPs (Schüller et al. 2008), additionally this experiment supports the first aim to specify the role of β1-Integrin in CGNPs.

Furthermore, to learn more about the clinical relevance of β1-Integrin in medulloblastomas, the expression levels of *ITGB1 (*human β1-Integrin gene) was analyzed with relative, quantitative, real-time, reverse transcriptase (RT)–PCR in human medulloblastoma samples and this data were correlated with clinical parameters.

2. Material and Methods

2.1. Mouse Lines, Genotyping, and Analysis of Recombination

Itgb1$^{Fl/Fl}$ (Raghavan et al. 2000), SmoM2$^{Fl/Fl}$ (Mao et al. 2006), and hGFAP-cre mice (Zhuo et al. 2001) were obtained from The Jackson Laboratory (Bar Harbour, ME).

2.1.1. *Itgb1$^{Fl/Fl}$* mice

A constitutive gene knockout of β1-Integrin in mice leads to a lethal phenotype, because of its essential relevance for the development (Fassler and Meyer 1995; Gaveriaux-Ruff and Kieffer 2007). To disrupt the function of β1-Integrin only in a special cellular compartment to a determined time, a Cre-LoxP system was used to generate conditional knockout mice.

Therefore in the β1-Integrin gene, of the used mouse mode,l two LoxP sites are flanking Exon 3. This is called a floxed β1-Integrin gene (Figure 4).

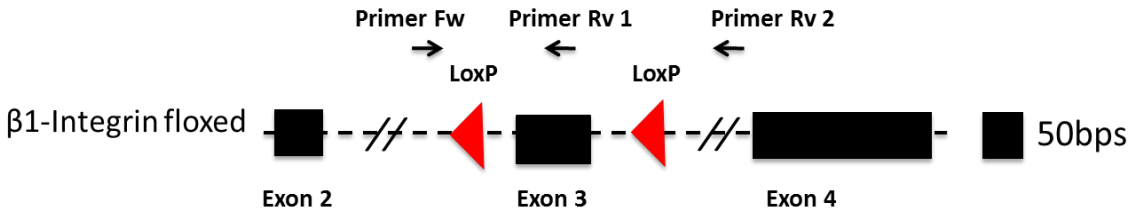

Figure 4 Schematic structure of the floxed segment within the β1-Integrin gene in mice obtained from Jackson Laboratory. Exon 3 flanked by LoxP sites (red triangles). The arrows represent the primers to detect the Cre-recombination.

The loxP sites are short sequences of 34bps (base pairs) and show the Cre recombinase where to recombine. As a consequence of the recombination the gene segment between the two LoxP sites will be recombined and removed.

The Cre-mediated recombination of the *Itgb1* (mouse β1-Integrin gene) allele was analyzed by PCR using the following primers in a triple-primer procedure (Figure 4):

Itgb1 Fw (Forward), 5'-CGCAGAACAATAGGTGCTGAAATTAC-3',

Itgb1 Rv (Reverse) 1, 5'-GCCCTGCTTGTATACATTCTCCG-3',

Itgb1 Rv 2, 5'-CCACAACTTTCCCAGTTAGCTCTC-3'.

2.1.2. *Math1*-cre mice

The Cre recombinase is a tool used in targeted gene modification, it was first discovered and isolated from bacteriophage P1 (Sauer and Henderson 1988; Sternberg et al. 1981). The expression of the Cre recombinase is spaciotemporarily controlled by a promotor or an enhancer sequence (Gaveriaux-Ruff and Kieffer 2007). To specifically express the Cre recombinase in CGNPs, the 1.4kB upstream *Math1* enhancer element was used in the mouse model for this study (Matei et al. 2005; Schüller et al. 2007). The *Itgb1*$^{Fl/Fl}$ mice were crossed with *Math1*-cre mice to generate *Math1-cre::Itgb1*$^{Fl/F}$ and *Math1*-cre::*Itgb1*$^{Fl/+}$ to delete β1-Integrin selectively in CGNPs.

2.1.3. *SmoM2*$^{Fl/Fl}$ mice

Smoothened (Smo) is a transmembrane protein of the Sonic hedgehog (Shh) signaling pathway. If the Sonic hedgehog pathway functions properly during developmental processes, the Sonic hedgehog protein binds to an extracellular domain of the Patched receptor and releases its inhibition of the Smoothened protein. This activation of Smoothened results in the expression of several genes (Ho and Scott 2002; Schüller et al. 2007), which are also responsible for the regulation of CGNPs and their proliferation in the EGL (Wechsler-Reya and Scott 1999)(Figure 5).

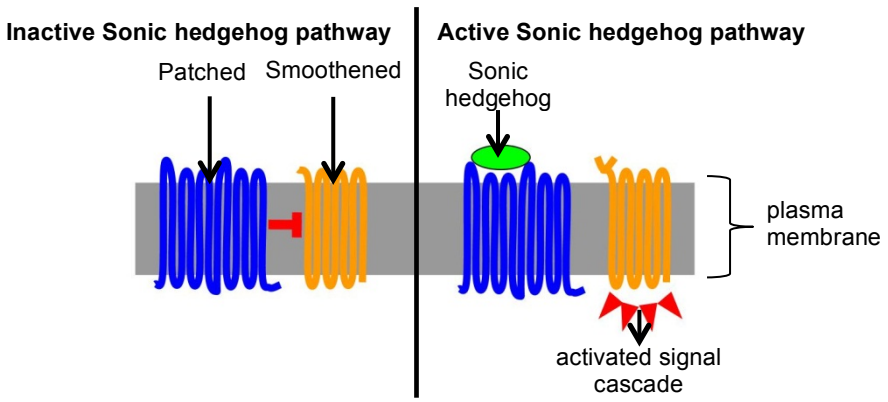

Figure 5 (modified from (Weinberg 2007)) The sonic hedgehog pathway. The Patched receptor (blue) is a transmembrane protein with 12 membrane-spanning domains and inhibits the Smoothened receptor (orange), a second transmembrane receptor spanning the membrane seven times. If the sonic hedgehog (Shh)(light green) protein binds to the extracellular domain of the Patched receptor, it will relieve the inhibition of Smoothened. Smoothened then sets in motion a intracellular signal cascade, including transcription factors to activate target genes of Sonic hedgehog for proliferation.

If Smoothened carries a point mutation with the subsequent amino acid exchange W539L, the Smoothened protein and the sonic hedgehog pathway will be activated

autonomously and constitutive, this mutation is called the *SmoM2* (Mao et al. 2006; Xie et al. 1998).

A stop sequence, consisting of a polyadenylation casette (4xpA) is located upstream of *SmoM2* to avoid ubiquitous expression of this oncogene. Additionally this stop sequence is floxed to remove it by a Cre recombinase and control the expression of *SmoM2* conditionally (Figure 6).

Figure 6 Simplified schematic structure of the *SmoM2*$^{FL/FL}$ gene, with a floxed STOP (red box) sequence, consisting of a polyadenylation cassette upstream of the *SmoM2* (yellow box) oncogene.

Itgb1$^{Fl/Fl}$ mice, *Math1*-cre mice and *SmoM2*$^{Fl/Fl}$ mice were crossed according to the following scheme in Figure 7 to receive the *Math1-cre::SmoM2*$^{Fl/+}$ (control mice), *Math1-cre::Itgb1*$^{Fl/Fl}$ *SmoM2*$^{Fl/+}$ and *Math1-cre::*Itgb1$^{Fl/+}$*SmoM2*$^{Fl/+}$ mice.

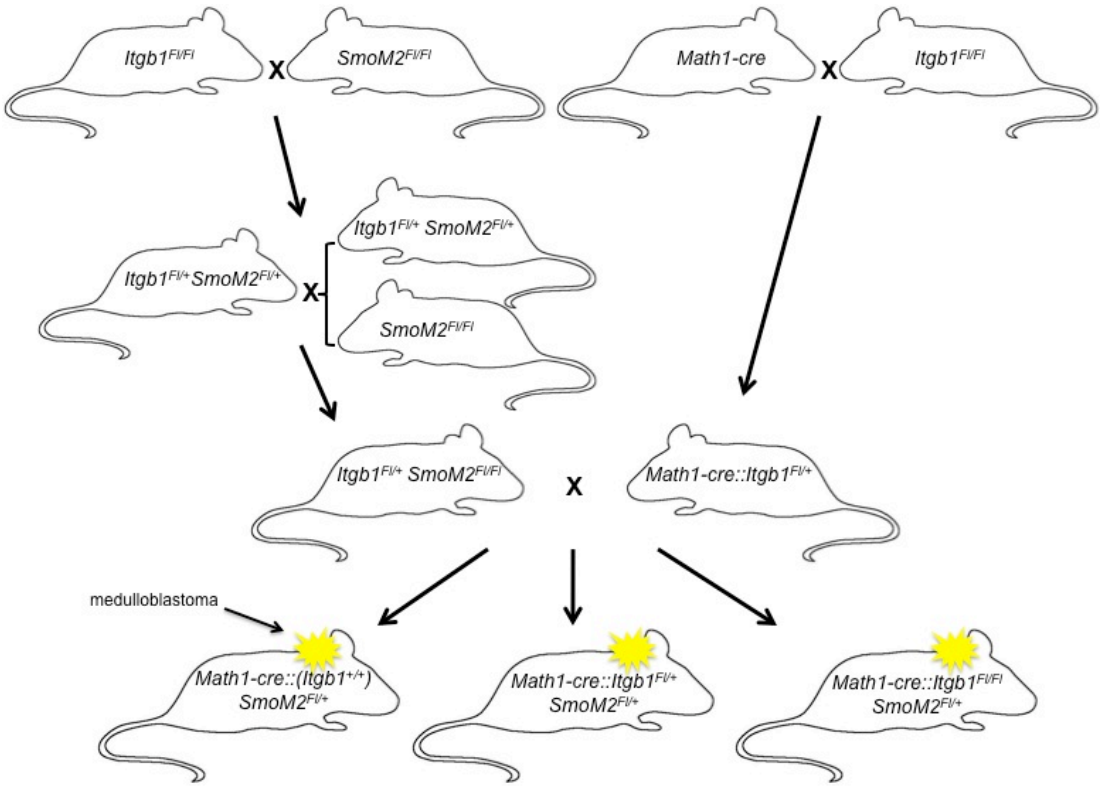

Figure 7 Crossing scheme to receive *Math1-cre::SmoM2*$^{Fl/+}$ (control mice), *Math1-cre::Itgb1*$^{Fl/Fl}$ *SmoM2*$^{Fl/+}$ and *Math1-cre::*Itgb1$^{Fl/+}$*SmoM2*$^{Fl/+}$ mice.

For genotyping to proof the *SmoM2* allel and the wild type allel the following primers were used:
SmoM2 Fw, 5'- AAGTTCATCTGCACCACCG -3';
SmoM2 Rv, 5'-TCCTTGAAGAAGATGGTGCG -3';
Smo WT Fw, 5'-GGAGCGGGAGAAATGGATATG-3';
Smo WT Rv, 5'-CGTGATCTGCAACTCCAGTC -3' (Jeong et al., 2004; Soriano, 1999).

Mice suspected to develop a tumor according to their genotype with a *Math1*-cre plus a *SmoM2* allele, were observed for neurological symptoms and general weakness twice a day.

2.1.4. *hGFAP-cre* mice:

The human glial fibrillary acidic protein (*hGFAP*) promotor sequence was used to control the expression of the Cre recombinase in glial cells of the transgenic mice in this study (Zhuo 2001). The genotyping for these mice was done with this primers:
hGFAP-cre Fw 5'-ACTCCTTCATAAAGCCCTCG-3',
hGFAP-cre Rv 5'-ATCACTCGTTGCATCGACCG-3' (Zhuo et al. 2001). *hGFAP-cre* mice were crossed with *Itgb1*$^{Fl/Fl}$ mice to generate *hGFAP*-cre*::Itgb1* $^{Fl/Fl}$ and *hGFAP*-cre*::Itgb1*$^{Fl/+}$ mice.

2.1.5. Genotyping of mice

To identify the genotype of the mice aforementioned, a PCR was performed of genomic DNA extracted from mouse tail biopsies. 300 µl lyses buffer (100 mM Tris pH 8.5; 5 mM EDTA pH 8.0, 0.2% SDS (filtered), 200 mM NaCl) and 10 µl of Proteinase K (10mg/ml) were added to the mouse tail. This solution digested the mouse tail for at least 2 hours at 55°C. After this time, the equal volume of UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v; from Invitrogen) was added and shaken for 15 minutes. Then this solution was fuged with 13.000 x g for 15 min at room temperature, to extract the top layer containing the DNA in a new tube. To precipitate the DNA, 50 µl 3 M sodium acetate (pH 5.2) and 1 ml of cold 95% ethanol were added. Than this solution was centrifugated with 13.000 x g at 4°C for 10 min. Liquid was discarded and the remaining liquid was removed with pipeman. The pellet was washed with 800 µl 70% Ethanol and mixed carefully and fuged another time with 13.000 x g at

4°C for 10 minutes. Then DNA pellet was dissolved in 100 µl DEPC (Diethylpyrocarbonate) water and stored at 4°C.

2.2. Patients and Tumor Samples

Overall, 37 surgical tumor samples from patients with medulloblastoma were analyzed. Patients included 22 males (58.5%) and 15 females (41.5%). They were treated at the University Hospital of Munich, Göttingen, Bremen, Hanover and Münster (all Germany). The majority of the patients with medulloblastoma were enrolled in the prospective multicenter trials of the German Society of Pediatric Hematology and Oncology. The median age of medulloblastoma patients was 7 years (range 0.7–39.7 years). The follow-up of survivors was 35.3 months (range 2.5–175.6 months). Up to the date of September 1st 2010, eleven of these patients succumbed the disease and 26 of them survived. The study included 1 anaplastic medulloblastoma, 22 medulloblastomas of classic histology, 10 medulloblastomas of desmoplastic histology, and 4 medulloblastomas with extensive nodularity. Detailed patient characteristics are given in Table 1. The tumors were diagnosed by standard light microscopic examination of hematoxylin and eosin-stained sections and silver stains. In accordance with the WHO brain tumor classification (Louis et al. 2007) the tumors were diagnosed independently by at least two neuropathologists. Autopsy material of normal cerebella was obtained from patients, who gave their informed consent. Patient age ranged between 18 and 67 years for adult cerebella, and between 22 weeks of gestation and 6 months for developing cerebella. None of these patients died from a central nervous system disorder or showed neuropathological abnormalities on histological examination. All tissue samples were either immediately fixed in formaldehyde and embedded in paraffin or snap-frozen and stored at -80°C until use.

Sample number	Histological subtype	Sex	Age [years]	Follow up [months]	Status	Relative *ITGB1* mRNA expression
1	anaplastic	f	10.4	5.3	NED	0.28
2	classic	m	2.4	21	DOD	4.9
3	classic	f	3.6	21.1	DOD	0.04
4	classic	f	3.7	5.4	NED	5.88
5	classic	m	4.1	35.4	NED	0.01
6	classic	m	4.9	75.7	DOD	0.23
7	classic	m	5.4	135.6	NED	6.87
8	classic	f	5.5	28.3	DOD	5.93
9	classic	m	5.5	137.5	NED	1.59
10	classic	m	5.9	2.5	NED	2.06
11	classic	m	6.2	22.2	DOD	22.42
12	classic	m	6.7	33.4	NED	7.61
13	classic	m	7.0	113.2	NED	10.66
14	classic	m	7.9	24.1	DOD	8.79
15	classic	m	8.5	36.5	DOD	7.38
16	classic	m	9.3	56.0	NED	7.06
17	classic	m	9.9	79	NED	8.26
18	classic	m	10.6	23.7	NED	2.23
19	classic	m	11.6	27.3	NED	0.5
20	classic	m	16.5	27.2	NED	2.03
21	classic	m	22.3	35.3	NED	7.76
22	classic	f	29.3	19.5	NED	5.11
23	classic	m	39.7	24.4	DOD	3.22
24	desmoplastic	f	1.6	66.9	NED	1.44
25	desmoplastic	f	2.2	128.0	NED	3.87
26	desmoplastic	m	2.5	7.0	DOD	0.02
27	desmoplastic	m	7.5	14.2	DOD	3.65
28	desmoplastic	f	14.3	62.7	DOD	7.74
29	desmoplastic	m	22.8	47.3	NED	8.10
30	desmoplastic	f	25.5	75.5	NED	0.03
31	desmoplastic	f	26.5	46.0	NED	4.97
32	desmoplastic	f	30.5	46.4	NED	1.67
33	desmoplastic	f	30.7	17.7	NED	3.08
34	extensively nodular	f	0.7	107.3	NED	0.01
35	extensively nodular	f	2.2	175.6	NED	2.47
36	extensively nodular	m	2.7	5.6	NED	2.46
37	extensively nodular	f	2.9	78.4	NED	2.59

Table 1 : Clinical data of patients with medulloblastoma. DOD, Death of disease; NED, No evidence of disease.

2.3. RNA Extraction and Real-Time PCR

Most cDNA probes of human medulloblastoma samples were obtained from Julia Pöschl, who extracted the RNA and performed reverse transcription according to the following protocols.

To extract total RNA, 20-30 of 10 µm thick slices of snap frozen tissue were gently homogenized with the pipette in 750 µl of TRIzol® reagent (Invitrogen). Then this homogenized tissue was incubated at room temperature for 5 min, subsequently 150 µl of Chloroform were added and the suspension was shaken for 15 sec and another time incubated for 3 min at room temperature. Through centrifugation with 14.000 x g for 15 min, the suspension was separated into three layers the undermost layer contained the DNA and TRIzol® reagent, the middle layer contained protein and cell detritus and the top layer contained RNA and Chloroform. The top layer (of approximately 350 µl) was transferred by pipette into a new tube. To precipitate the RNA, 375 µl of Isopropanol were added, and then it was briefly mixed, incubated for 10min at room temperature and afterwards fuged with 14.000 x g. The supernatant was discarded and the pellet was washed with 750 µl of Ethanol, then again the solution was centrifuged with 7.500 x g at 4°C. The Ethanol was discarded and the rest was removed with the pipette. Then the RNA pellet was dissolved in 24 µl of DEPC water. To determine the concentration of the RNA in the solution, NanoDrop 3300 was used (Thermo Fisher Scientific).

Subsequently total RNA was treated with RQ1 RNase-Free DNase (Promega), to remove the remaining DNA fragments. According to the protocol firstly the digestion reaction was prepared containing: 8 µl RNA (in DEPC water), 1 µl RQ1 RNase-Free DNase 10x Reaction Buffer, 1u RQ1 RNase-Free DNase was added per 1 µg of RNA. This solution was mixed and incubated at 37°C for 30 min, then 1 µl of RQ1 DNase Stop Solution was added to terminate the reaction. The following incubation at 65°C for 10 minutes inactivated the DNase.

SuperScript® II Reverse Transcriptase (Invitrogen) was used to run reverse transcription for human samples. Each reaction consisted of: Up to 4 µg total RNA in up to 8 µl, 1µl of 10 mM dNTP mix, mixture of 0.5 µl Oligo $(dT)_{15}$ primers and 0.5 µl random hexamers (50 ng/µl), this solution was filled up to 10 µl with DEPC-treated water. The RNA/primer mixture was incubated at 65°C for 5 min then it was placed on ice for at least 1 min. In a different tube the 2x reaction mix; per run was prepared in the following order: 2 µl 10x RT Puffer, 4 µl 25m M $MgCl_2$, 2 µl 0.1 M DTT, 1 µl RNaseOUT™ (40 U/µl) Recombinant Ribonuclease Inhibitor. 9 µl of this 2x reaction mix were added to each RNA/primer mixture and mixed gently, then it was incubated at room temperature for 2 min. To each tube 1 µl of SuperScript® II Reverse

Transcriptase was added and incubated at room temperature for 10 min, then it was incubated at 42°C for 50 min and the reaction was terminated at 70°C for 25 min and subsequently chilled on ice. Then the solution was collected briefly by centrifugation, 1 µl of RNase H was added to each sample and incubated for 20 min at 37°C. Then the samples were stored at -20°C until their use for PCR.

The real-time relative quantitative reverse transcriptase (RT)-PCR experiments were performed at the LightCycler® 480 (Roche) on 96 multiwell-plates with the LightCycler® 480 SYBR Green I Master reagents (Roche). When SYBR Green I dye binds to double-stranded DNA, the fluorescence signal of SYBR Green I dye in this complex increases significantly. With progressive amplification of the specific DNA segment during PCR reaction also the fluorescence increases, which is measured after every cycle of PCR at the end of the elongation.

The SYBR Green I Master Mix for LightCycler®480 included already a FastStart Taq DNA Polymerase and the SYBR Green I dye (Roche), from this mix 5 µl were used for each reaction. Furthermore it was added 1 µl Fw Primer and 1 µl of Rv Primer and 3µl of the cDNA. The LightCycler® 480 was programmed for PCR as follows:

1. Incubation at 95°C for 5min
2. Denaturation at 95°C for 10 seconds
3. Annealing at 58°C for 10 seconds
4. Elongation at 72°C for 15 seconds

} These steps were repeated 45 times

In this study the *ITGB1* (human β1 Integrin) was the target gene, the aim was to measure the level of *ITGB1* expression in tissue of human medulloblastoma. To perform relative quantification of the expression of the target gene Beta-2 microglobuline was used as housekeeping gene, which is expressed constitutively in every sample and independent from pathological changes at the same level, . Additionally, every round of measurement the same calibrator sample was used to normalize the different rounds. Every measurement was done three times. The appropriate primers for this were found with Primer 3 software experiment. Sequences were as follows for the indicated genes:

ITGB1 FW 5'-TGGGCTTTACGGAGGAAGTA-3';

ITGB1 RV 5'-CTACCAACACGCCCTTCATT-3',

B2M FW 5'-TGTCTTTCAGCAAGGACTGG-3';

B2M RV 5'- GATGCTGCTTACATGTATCG-3'.

For each primer pair, melting curves were conducted after the amplification with LightCycler480® software and agarose gel electrophoresis were performed to

determine the presence of a single amplification product. To correct the efficiency for each set of primers, a standard curve was created. The efficiency of the *B2M* primer pair was 1.887 and for *ITGB1* primer pair was 1.875.

The calibrator normalized relative ratio was calculated the following way:

Normalized Ratio=$E_T{}^{CpT(C)-CpT(S)}$x$E_R{}^{CpR(C)-CpR(S)}$

CpT/CpR: Cycle number at target/reference detection threshold (crossing point); E_T/E_R: Efficiency of target/reference amplification; T: Target; R: Reference; S: Unknown sample; C: Calibrator

2.4. Histology and Immunohistochemistry

For all histological methods, brains were dissected, fixed overnight in 4% paraformaldehyd at 4°C, embedded in paraffin, and sectioned at 5 µm according to standard protocols. Hematoxylin and eosin (H&E) stains or Bielschowsky silver impregnations were performed according to standard protocols. For immunohistochemistry, paraffin sections were subjected to heat antigen retrieval at 100°C for 20 min in 10 mM sodium citrate buffer for all antibodies. Staining was performed using the HRP/DAB staining system (DAKO) according to the manufacturer's specifications. Primary antibodies were NeuN (Chemicon), Caspase3 (Cell Signaling Technology), Ki67 (DAKO), PCNA (DAKO), phospho-Histone H3 (pHH3, Cell Signaling Technology), Pax2 (Zymed), Pax6 (Developmental Studies Hybridoma Bank), Sox2 (Chemicon), Calbindin (Sigma), GFAP (DAKO), Cre recombinase (Covance), Zic1 (gift from R. Segal) and S100 (DAKO). All histological photomicrographs were taken digitally using an Olympus BX50 microscope in combination with the ColorView (Soft Imaging System).

2.5. Western Blot analysis

Proteins were extracted from tissue samples, lysis was performed on ice for 30 minutes in 500 µl of ice-cold lysis buffer containing 50 mmol/L Tris-HCl (pH 8), 120 mmol/L NaCl, 0.5% Nonidet® P-40 (Sigma), 1 mmol/L phenylmethyl sulfonyl fluoride (Sigma), 100 U/ml aprotinin (Calbiochem), and 0.1 mol/L NaF. Debris was removed by centrifugation for 20 minutes at 12,000 x g and at 4°C (Hartmann et al. 2005). Samples

were separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis and blotted on nitrocellulose. To document equal loading of the gels, Ponceau staining of the membranes was performed. Blocking was performed with 10% nonfat dry milk (Bio-Rad) in Tris-buffered saline containing 0.05% Tween 20 and 20% horse serum for 2 hours at room temperature. Subsequently, membranes were incubated with rabbit β1-Integrin antiserum overnight (gift from Dr. Staffan Johansson, Uppsala University, Sweden). An alkaline phosphatase-coupled goat anti-rabbit antibody together with the CDP-Star solution (Roche) was used for detection. Quantifications were normalized to individual Actin levels for each sample.

2.6. Statistical Analysis

All obtained results were analyzed using the Prism4 software (Graph Pad). *In vivo* counting of phospho-histone H3-postive cells in the EGL was performed relating the total number of immunolabeled cells on each section to the overall length of the EGL. Results derived from counting three different sections of two animals for each genotype. Survival of patients and mice was analyzed using Kaplan–Meier survival curves and the Log-rank test was used to examine the significance of results. P-values < 0.05 were considered as significant. The unpaired t-test was applied to compare the means of two groups with assumed Gaussian distribution and equal variances. If variances were not equal, as confined by the F-test, or Gaussian distribution was not expected, the nonparametric Mann–Whitney test for unpaired data was used to compare the medians of two groups. For quantification of immunohistochemical reactivity for S100 and Cre in the Purkinje cell layer (PCL) and the molecular layer (ML), positive cells in lobules III and V were counted in three sagittal cerebellar sections per mouse for three different animals per genotype. The PCL was defined as the area from 5 µm below the bottom to 5 µm above the top of Purkinje cell somata. The number of immunoreactive cells was counted separately and expressed as cells per mm PCL.

3. Results

3.1. Expression of β1-Integrins in Cerebellar Granule Neuron Precursors

β1-Integrins in *Nestin*-positive precursors were previously found to regulate the formation of cortical structures in the brain (Graus-Porta et al. 2001). The deletion of β1-Integrin in *Nestin*-positive neural precursors in conditional knockout mice results in a decrease in size and perturbed cortical lamination of the cerebellum. It should be mentioned that *Nestin* is expressed in all kinds of neural precursors. To address the specific role of β1-Integrins in CGNPs for the formation of the cerebellar cortex, the *Itgb1* gene was conditionally inactivated by using a *Math1-cre* line, which is known to recombine CGNPs, but no other cell types of the cerebellar cortex (Machold and Fishell 2005; Schüller et al. 2007; Wang et al. 2005). In a first step, it was analyzed whether β1-Integrins have indeed been deleted from CGNPs in this mouse model. Therefore genomic DNA from the EGL of paraffin sections was isolated and checked for the recombination of the *Itgb1* allele by semiquantitative PCR. As expected, the *Itgb1* allele has been recombined in the majority of cells in the EGL (Figure 8 A).

Furthermore, Western blot analysis of cerebellar tissue from *Math1-cre::Itgb1*$^{Fl/Fl}$ mice isolated at P7 revealed a reduction of almost 50% in β1-Integrin protein levels (Figure 8 B).

Bearing in mind whole cerebellum lysates which also contained *Math1*-negative cell types were used, it was reasoned that β1-Integrins have been deleted in the majority of *Math1-cre::Itgb1*$^{Fl/Fl}$ CGNPs.

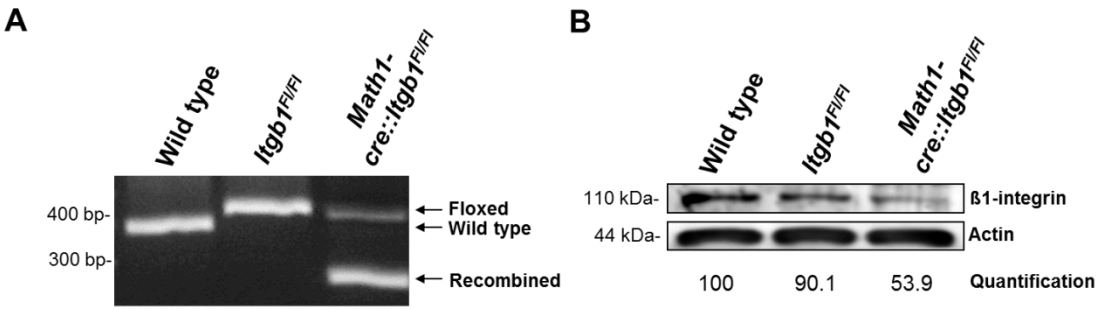

Figure 8 Inactivation of β1-Integrins in CGNPs of the cerebellum. PCR analysis of genomic DNA isolated from the external granule layer of wild type, *Itgb1*$^{Fl/Fl}$ and *Math1-cre::Itgb1*$^{Fl/Fl}$ mice at postnatal day 7 (P7) indicating the different *Itgb1* alleles (A). Western blot analysis for β1-Integrins in cerebella of indicated genotypes confirmed deletion of β1-Integrins in *Math1-cre::Itgb1*$^{Fl/Fl}$ mice (B).

To investigate the effect of β1-Integrin deletion in CGNPs, Hematoxylin and Eosin-stained sagittal sections were assessed of postnatal day 7 (P7) and adult

Math1-cre::Itgb1$^{Fl/Fl}$ mice to analyze the overall organization of the cerebellar cortex. As in *Itgb1*$^{Fl/Fl}$ mice that do not express Cre recombinase in CGNPs, cortical layers of the cerebellum in *Math1-cre::Itgb1*$^{Fl/Fl}$ mice appeared normal during development and at the adult stage (Fig. 9 A,B,D,E,G,H,J,K).

Immunohistochemical staining using antibodies against NeuN, a postmitotic neuronal marker, revealed that the differentiation of CGNPs into mature cerebellar granule neurons remained unaffected in *Math1-cre::Itgb1*$^{Fl/Fl}$ mice (Fig. 9 C,F,I,L). Furthermore, Caspase3 immunoreactivity displayed no differences with respect to the apoptotic activity in cerebella of *Itgb1*$^{Fl/Fl}$ and *Math1-cre::Itgb1*$^{Fl/Fl}$ mice at P7 (Fig. 9 M,Q). In both control and conditional knockout mice, only few apoptotic cells were visible, predominantly in the IGL and the prospective white matter. It has previously been proposed that β1-Integrins are essential for the proliferation and the ongoing expansion of the CGNP pool during development (Blaess et al. 2004). To address whether the deletion of β1-Integrins in CGNPs in vivo caused any proliferative defects that would not be visible on standard histology, the number of proliferating cells in the EGL at P7 was analyzed. This age was chosen, since it represents the phase of highest CGNP proliferation during cerebellar development (Wechsler-Reya and Scott 1999). However, immunostaining for proliferation markers such as Ki67 and proliferating cell nuclear antigen (PCNA) revealed no differences between *Math1-cre::Itgb1*$^{Fl/Fl}$ and *Itgb1*$^{Fl/Fl}$ mice (Fig. 9 N,R,O,S). Further antibodies against phosphorylated histone H3 (pHH3), expression of which marks cells in the M phase of the cell cycle, were used and analyzed its expression in the cerebellar EGL (Figure 9 P,T). Cell counts in histological sections confirmed that the EGL of *Math1-cre::Itgb1*$^{Fl/Fl}$ mice contained normal numbers of mitotic cells (Figure 9 U). In addition to that, primary cultured and Cre-IRES-GFP virus-transduced CGNPs from postnatal day 7 *Itgb1*$^{Fl/Fl}$ mice revealed no statistically significant differences with respect to BrdU-incorporation as compared with control cells that were transduced with IRES-GFP virus (Figure 9 V, this experiment was performed by Pierfranesco Pagella). Thus, the acute deletion of β1-Integrins in CGNPs did not affect their proliferative activity in response to Sonic hedgehog. Taken together, the conclusion is that the expression of β1-Integrins in CGNPs is not required for the proper formation of the murine cerebellum.

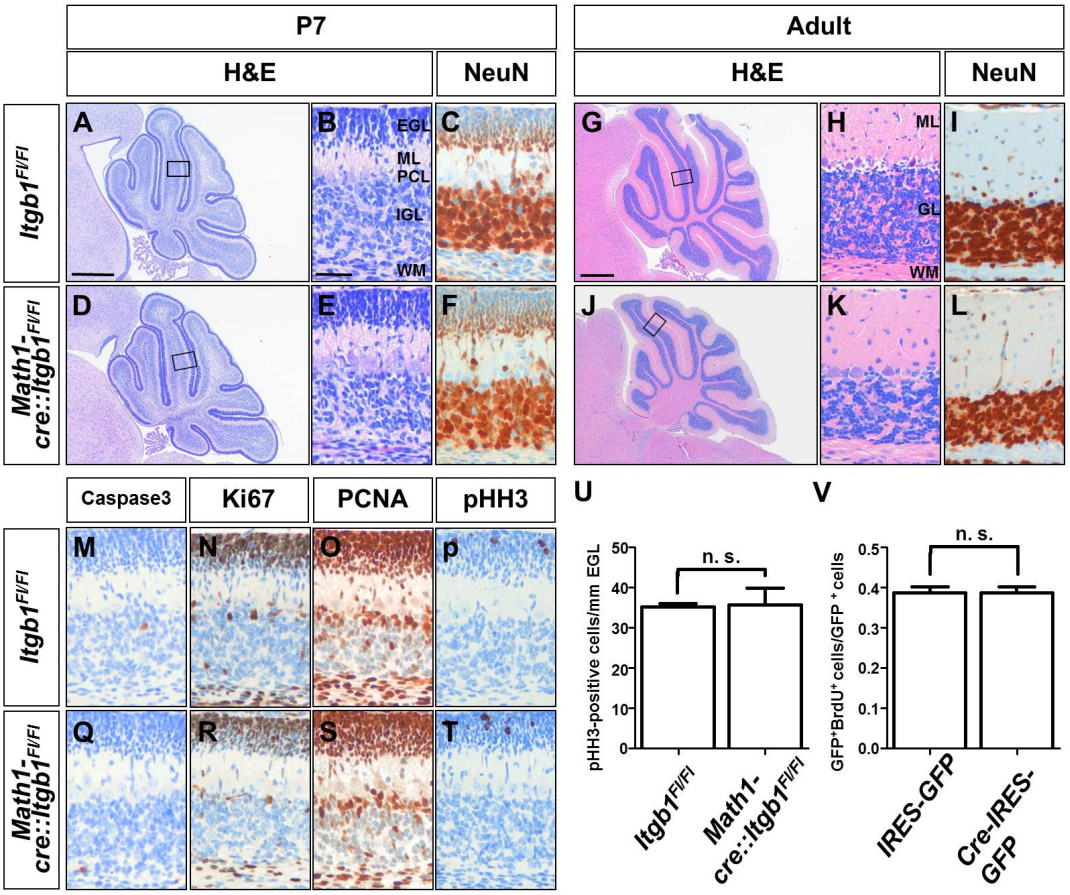

Figure 9 Inactivation of β1-Integrins in CGNPs of the cerebellum. Histological analysis of cerebella from *Itgb1*$^{FI/FI}$ and *Math1-cre::Itgb1*$^{FI/FI}$ mice did not show any differences at P7 or adult stages (C–T). Paraffin-embedded sections were stained with Hematoxylin-Eosin or antibodies specific for neuronal nuclei protein (NeuN), Caspase3, Ki67, proliferating cell nuclear antigen (PCNA), and phospho-Histone H3 (pHH3). Statistical analysis of pHH3-positive cells in the external granule layer of *Itgb1*$^{FI/FI}$ and *Math1-cre::Itgb1*$^{FI/FI}$ mice revealed no differences in proliferation (U). Acute deletion of β1-Integrins by Cre virus in cultured CGNPs of *Itgb1*$^{FI/FI}$ mice had no effect on proliferative activity in response to Sonic hedgehog (V this experiment was performed by Pierfrancesco Pagella). Boxed areas in A, D, G, and J are shown as higher magnifications in adjacent pictures. EGL, external granular layer; IGL, internal granular layer; ML, molecular layer; PCL,Purkinje cell layer; WM, white matter; n. s., not significant. Scale bars: 500 µm (in A for A, F; in G for G, J), 25 µm (in B for B, C, E, F, H, I, K-T).

3.2. Expression of β1-Integrin in Shh-induced mouse medulloblastoma and human medulloblastoma

Medulloblastoma, the most common malignant brain tumor in childhood, can be divided into four different subgroups depending on their gene expression profile and there is evidence that subtypes of medulloblastoma have distinct developmental origins (Gibson et al. 2010; Taylor et al. 2012). Medulloblastoma with activated Wnt pathway may arise outside of the cerebellum from cells of the dorsal brainstem (Gibson et al. 2010), whereas Shh-associated tumors are suggested to arise from the CGNP population of the cerebellum (Schüller et al. 2008). Given the previously reported importance of β1-Integrins in tumorigenesis and cerebellar development (Graus-Porta et al. 2001; White et al. 2004), β1-Integrin subunit was conditionally deleted from tumors in *Math1-cre::SmoM2*$^{Fl/+}$ mice that served as a model for CGNP-derived medulloblastoma (Schüller et al. 2008). In such mice with a *Math1-cre::Itgb1*$^{Fl/Fl}$*SmoM2*$^{Fl/+}$ genotype, Cre expression driven by *Math1* promoter sequences was supposed to delete β1-Integrins in CGNPs, while the Shh pathway is constitutively activated through the oncogenic *SmoM2* mutation that is expressed under control of the same promoter. To estimate whether the deletion of β1-Integrins had substantial impact on the survival of *Math1-cre::SmoM2*$^{Fl/+}$, a Kaplan–Meier analysis was first performed to compare the survival of *Math1*-cre::*SmoM2*$^{Fl/+}$ (n = 21), *Math1-cre::Itgb1*$^{Fl/+}$*SmoM2*$^{Fl/+}$ (n = 16), and *Math1-cre::Itgb1*$^{Fl/Fl}$*SmoM2*$^{Fl/+}$ mice (n = 40). However, there were not detected any statistically significant differences in the overall survival of these mice (Figure 10 A). PCR analysis from DNA of tumor tissue confirmed that the *Itgb1* allele had successfully been recombined both in *Math1-cre::Itgb1*$^{Fl/+}$*SmoM2*$^{Fl/+}$ and in *Math1-cre::Itgb1*$^{Fl/Fl}$*SmoM2*$^{Fl/+}$ mice (Figure 10 B). Moreover, protein levels of β1-Integrins were clearly reduced in tumors of *Math1-cre::Itgb1*$^{Fl/+}$*SmoM2*$^{Fl/+}$ mice and almost absent in tumors of *Math1-cre::Itgb1*$^{Fl/Fl}$ *SmoM2*$^{Fl/+}$ mice (Figure 10 C). Histological analysis of the tumors did not reveal any differences in *Math1-cre::Itgb1*$^{Fl/+}$*SmoM2*$^{Fl/+}$ or *Math1-cre::Itgb1*$^{Fl/Fl}$*SmoM2*$^{Fl/+}$ mice as compared with control mice with respect to overall morphology and immunoreactivity for Ki67 (Figure 10 D–L). Quantification of the proliferative activity did not reveal any significant differences between the indicated genotypes (Figure 10 M).

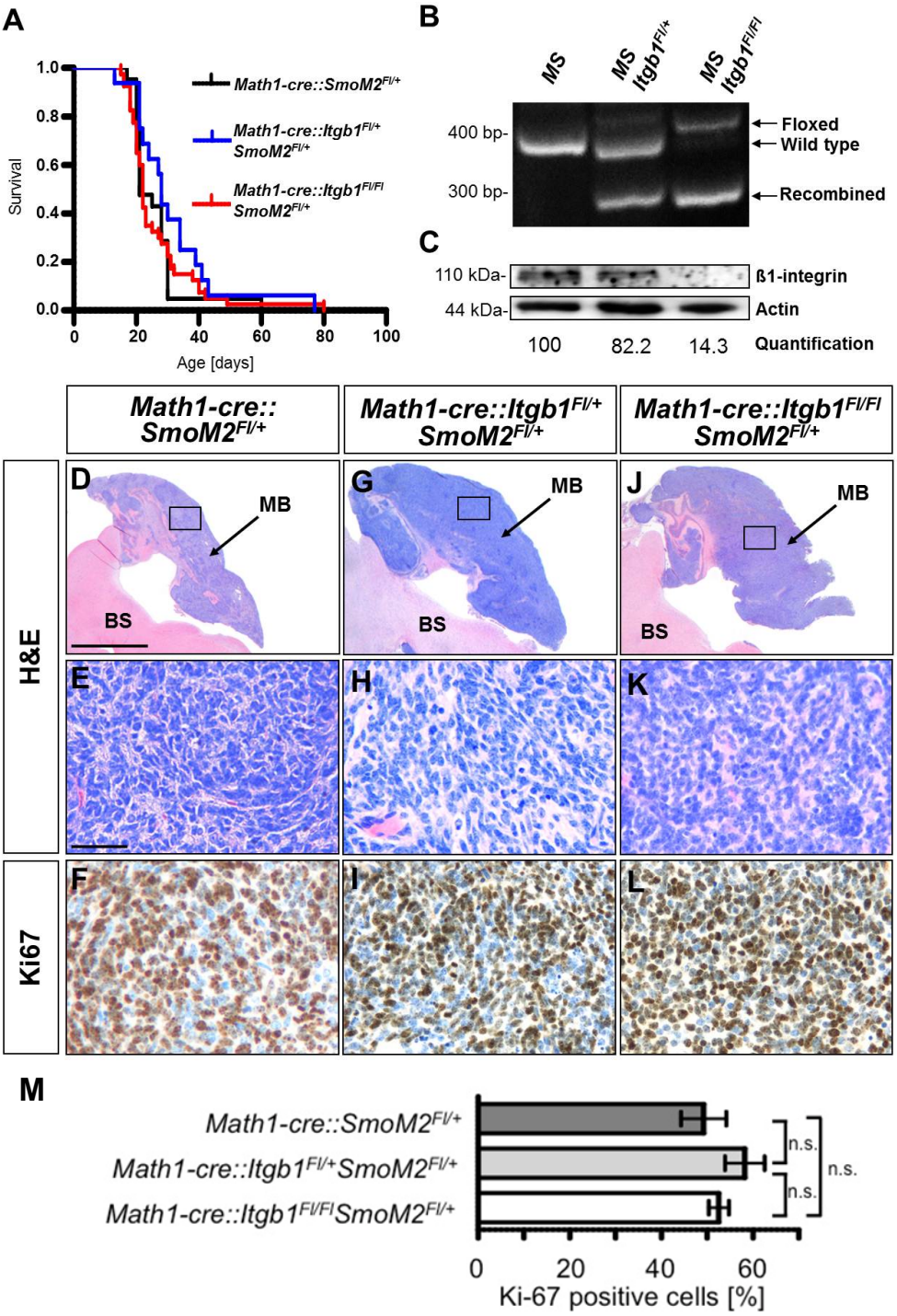

Figure 10 Analysis of Itgb1 in medulloblastoma. Kaplan–Meier curves for mice of the three indicated genotypes revealed no statistically significant differences in survival (A). Semiquantitative PCR analysis of DNA from murine medulloblastoma confirmed the successful recombination of the Itgb1 allele (B). Expression levels of βIntegrin1-Integrin protein in indicated medulloblastoma tissues confirmed β1-integrin deletion in *Math1-cre::Itgb1*$^{Fl/Fl}$*SmoM2*$^{Fl/+}$ mice (C). Histology with low and high magnification and expression of Ki67 visualized by immunohistochemistry of *Math1-cre::SmoM2*$^{Fl/+}$, *Math1-cre::Itgb1*$^{Fl/+}$*SmoM2*$^{Fl/+}$, and *Math1-cre::Itgb1*$^{Fl/Fl}$*SmoM2*$^{Fl/+}$ mice showed no differences (D–M). Relative expression of ITGB1 in human developing cerebellum (DCB), adult cerebellum (ACB), desmoplastic medulloblastoma (DMB), and classic medulloblastoma (CMB) revealed high expression of ITGB1 in medulloblastomas and developing cerebella in comparison to adult cerebella (N). No statistically significant differences between the histological subtypes, CMB and DMB, were noted with respect to ITGB1 expression. Overall survival rates of medulloblastoma patients (n = 37) grouped according to those with expression levels of ITGB1 below the median and above the median were similar (P 5 0.2, O). MB, medulloblastoma; BS, brainstem; n. s., not significant. Scale bars: 3 mm (in D for D, G, J); 50 μm (in E for E, F, H, I, K, L).

Last, the β1-Integrin-deficient tumors displayed normal expression with regard to Zic1 that labels cerebellar granule neurons and granule neuron-derived medulloblastomas (Figure 11 A–D).

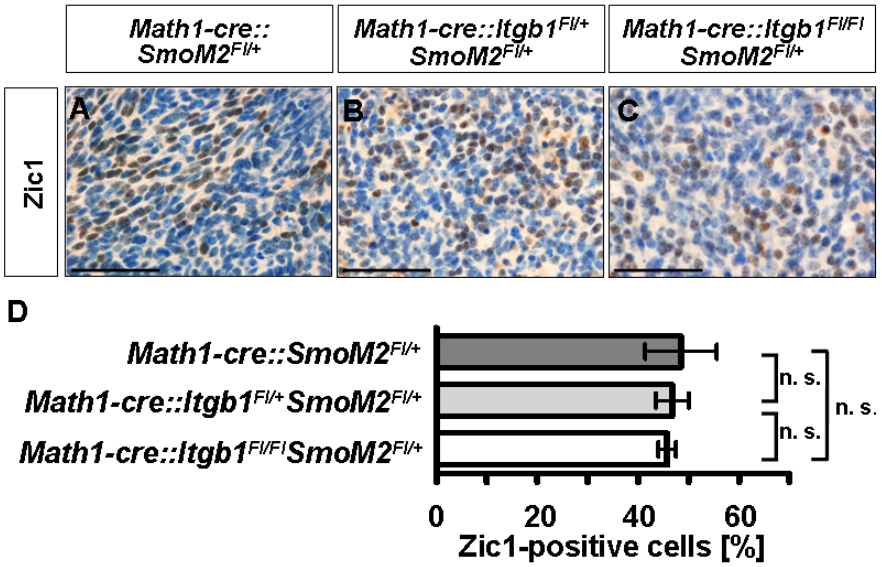

Figure 11 Expression of Zic1, a marker of neuronal differentiation, in Itgb1-deficient and control medulloblastoma of mice with the indicated genotype. Expression of Zic1 (A-C) show no differences in medulloblastoma from indicated mouse models. Quantification for Zic1-positive cells in medulloblastomas from *Math1-cre::SmoM2$^{Fl/+}$, Math1-cre::Itgb1$^{Fl/+}$SmoM2$^{Fl/+}$ and Math1-cre::Itgb1$^{Fl/Fl}$SmoM2$^{Fl/+}$* mice (D). Scale bars: 50 µm.

Since the mouse model of Shh-associated medulloblastoma used for this study, did most likely not fully reflect the situation in all molecular subtypes of human medulloblastoma, real-time RT-PCR was used to analyze the relative expression of human *Integrin β1 gene* (*ITGB1*) in human samples of normal cerebella (n = 8) and medulloblastoma (n = 37) and subsequently its correlation with the patients' survival was investigated.

In comparison to adult cerebella, *ITGB1* was highly expressed during development and in medulloblastomas (P = 0.03 and P = 0.04, respectively, Figure 12). However, there was no significant difference in *ITGB1* expression between desmoplastic (n = 10) and classic (n = 22) medulloblastomas, the two major histological tumor subtypes (P = 0.13, Figure 12).

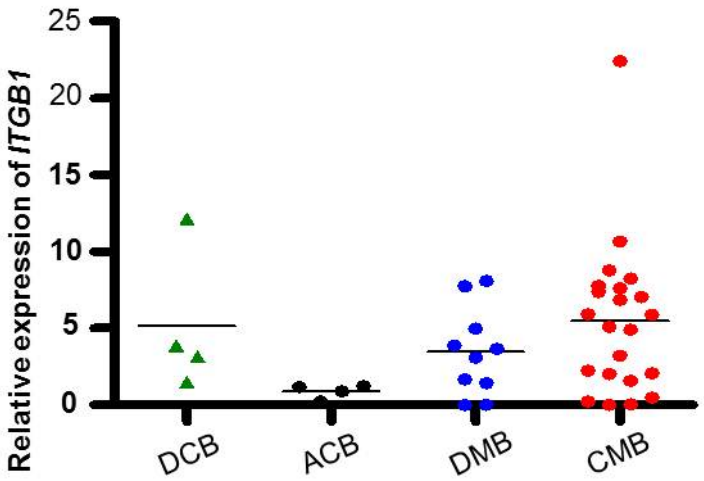

Figure 12 Relative expression of *ITGB1* in human developing cerebellum (DCB), adult cerebellum (ACB), desmoplastic medulloblastoma DMB), and classic medulloblastoma (CMB) revealed high expression of ITGB1 in medulloblastomas and developing cerebella in comparison to adult cerebella. No statistically significant differences between the histological subtypes, CMB and DMB, were noted with respect to ITGB1 expression.

To further support this finding, publicly available microarray data from an independent series of 62 medulloblastoma samples were reanalyzed (Kool et al. 2008) with regard to the *ITGB1* expression in different molecular subtypes of medulloblastoma. Molecular subtyping of medulloblastomas was performed according to a recent consensus that defined four main subtypes (Taylor et al. 2012). According to this data, *ITGB1* was expressed in all molecular subtypes of medulloblastoma, and there was no statistically significant difference regarding its expression levels (Figure 13).

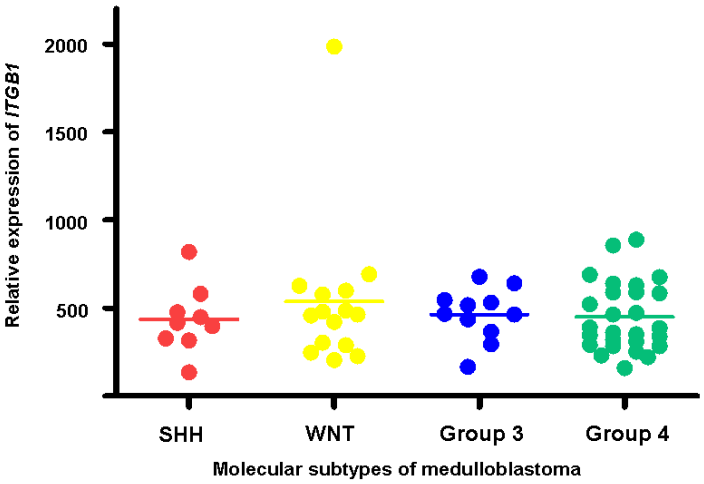

Figure 13 Relative expression of *ITGB1* in molecular subtypes of medulloblastoma molecular subgroups (Kool et al. 2008; Taylor et al. 2012). *ITGB1* is expressed in all molecular subtypes of medulloblastoma, but there is no statistically significant difference between the different subtypes regarding *ITGB1*.

Next, the survival of patients was analyzed according to the mRNA levels of *ITGB1*. For Kaplan–Meier analyses, patients were divided in two groups based on the median *ITGB1* expression of their tumors. As shown in Figure 14, the expression of *ITGB1* was not correlated with the patient's survival (P = 0.2). The conclusion of these results is that the expression of β1-Integrins in CGNPs was not essential for the formation of CGNP-derived medulloblastoma and had no impact on the survival of tumor-prone mice. Furthermore, *β1-Integrins* were highly expressed in human medulloblastomas, but their expression had no prognostic role in medulloblastoma patients.

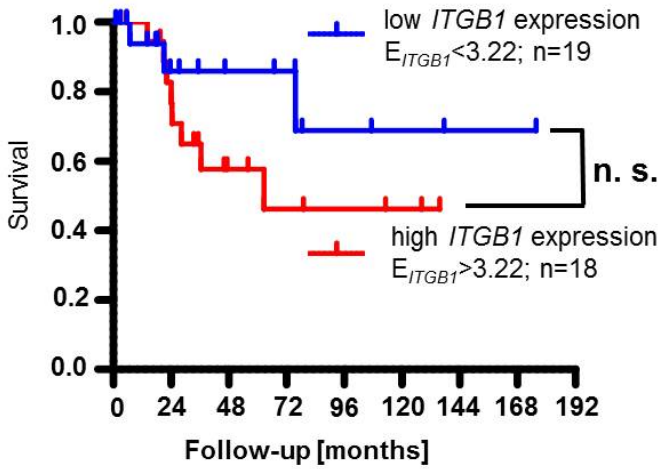

Figure 14 Overall survival rates of medulloblastoma patients (n = 37) grouped according to those with expression levels of *ITGB1* below the median and above the median were similar (P = 0.2). n. s.; not significant.

3.3. Expression of β1-Integrins in Glia of the cerebellum

Although conditional deletion of β1-Integrins under the control of *Nestin* promoter sequences had severe consequences for the development of the cerebellum, deletion of β1-Integrins in committed cerebellar granule neurons that express *Math1* did not affect cerebellar development or tumorgenesis. To further investigate, which cell types within the cerebellar cortex would require β1-Integrins for appropriate development, a *hGFAP*-cre mouse line was used for driving Cre-mediated deletion of β1-Integrins in glia of the postnatal cerebellum (Malatesta et al. 2003; Zhuo et al. 2001). Hematoxylin and Eosin-stained sections from 7-day-old *hGFAP*cre*::Itgb1*$^{Fl/Fl}$ mice revealed cerebella that were clearly smaller than those from *hGFAP*-cre:*Itgb1*$^{Fl/+}$ control mice (Figure 15 A,D). The depth of fissures as well as the size of individual lobules were decreased, and cerebellar lobules of *hGFAP*-cre*::Itgb1*$^{Fl/Fl}$ mice were fused together (Figure 15 B,C,E,F; arrow in F points to the fusion line of two adjacent lobules). In adult mutant cerebella, the overall size was still reduced and no clear fissures formed between adjoining lobules (Figure 15 G–L). The internal granular layer (IGL) was less prominent in size and displayed a rather washed-out and less confined structure. Moreover, some cerebellar granule neurons formed ectopia along the fusion line of adjacent lobules and on the cerebellar surface of *hGFAP*-cre*::Itgb1*$^{Fl/Fl}$ mice (Figure 15 I,L; arrow in L points toward cerebellar granule neurons ectopia on the fusion line of adjacent folia). To visualize the nerve fiber organization in the cerebellar cortex, a Bielschowsky silver impregnation was performed on cerebellar sections of *hGFAP*cre*:: Itgb1*$^{Fl/Fl}$ and control mice (Figure 15 M,N). Parallel fibers of the cerebellum of *hGFAP*-cre*::Itgb1*$^{Fl/Fl}$ mice appeared extenuated in terms of size and number as compared with control mice. Furthermore, parallel fibers did not display their natural orientation parallel to the cerebellar surface. They were arranged in a rather disoriented fashion throughout the ML (Figure 15 N). Taken together, the cerebellum of *hGFAP*-cre*::Itgb1*$^{Fl/Fl}$ mice was severely affected in terms of size, cortical lamination, and nerve fiber organization.

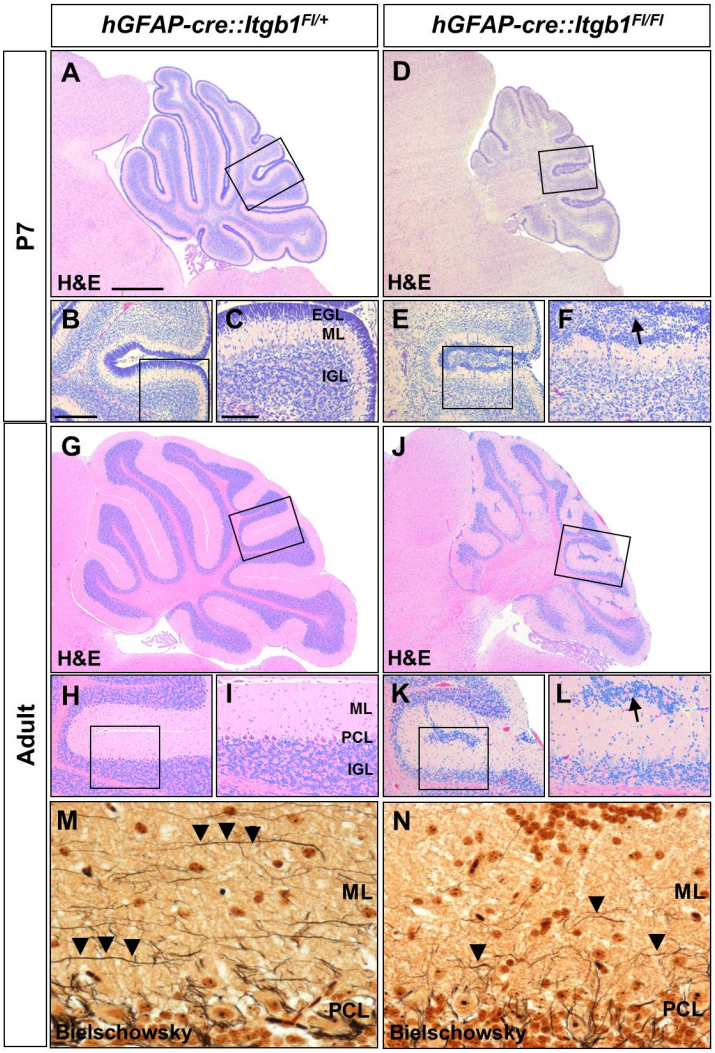

Figure 15 Cerebellar histology in *hGFAP-cre::Itgb1*$^{Fl/+}$ and *hGFAPcre::Itgb1*$^{Fl/Fl}$ mice. Cerebella were analyzed at postnatal day 7 (P7, A–F) and adult stage (G–P). Paraffin sections were stained with Hematoxylin-Eosin (H&E). Boxed areas are shown as higher magnifications in adjacent pictures. Arrows in (F) and (L) point to remnants of the external granule layer. Bielschowsky silver impregnation of nerve fibers reveals a gross disorganization of parallel fibers in the molecular layer of *hGFAP-cre::Itgb1*$^{Fl/Fl}$ mice (M, N).). EGL, external granule layer; IGL, internal granule layer; ML, molecular layer; PCL Purkinje cell layer. Scale bars: 2 mm (in A for A, D, G, J); 200 µm (in B for B, E, H, K); 100 µm (in C for C, F, I, L); 10 µm (M, N).

To further characterize the above described phenotype, immunohistochemistry was performed for several cellular markers on P7 and adult cerebella that were able to identify major neuronal cell types. Islands of ectopic cells that were stuck on the cerebellar surface were strongly positive for NeuN and Pax6, as early as P7 (Figure 16 A,B,F,G,K,L,P,Q), arguing that these cells are cerebellar granule neurons (Weyer and Schilling 2003) that were not able to migrate into the inner granule layer and prematurely differentiated in this position. Antibodies against Pax2 and Calbindin were then used to identify inhibitory interneurons and Purkinje neurons, respectively (Figure 16 C,D,H,I,M,N,R,S). Pax2-positive as well as Calbindin-positive cells were regularly detected in cerebella of P7 and adult *hGFAP*-cre*::Itgb1*$^{Fl/Fl}$ mice, suggesting that these

cell populations were properly specified and remained unaffected. Finally, expression of Ki67 revealed a normal proliferative activity in *hGFAP*cre*::Itgb1*$^{Fl/Fl}$ mice (Figure 16 E,J,O,T). However that cerebellar granule neuron ectopia did not proliferate, further supporting the hypothesis that these are mature cerebellar granule neurons. It is well known that cerebellar granule neurons of the external granular layer of the cerebellum migrate along Bergmann glial fibers into the inner granular layer (Hatten 2002).

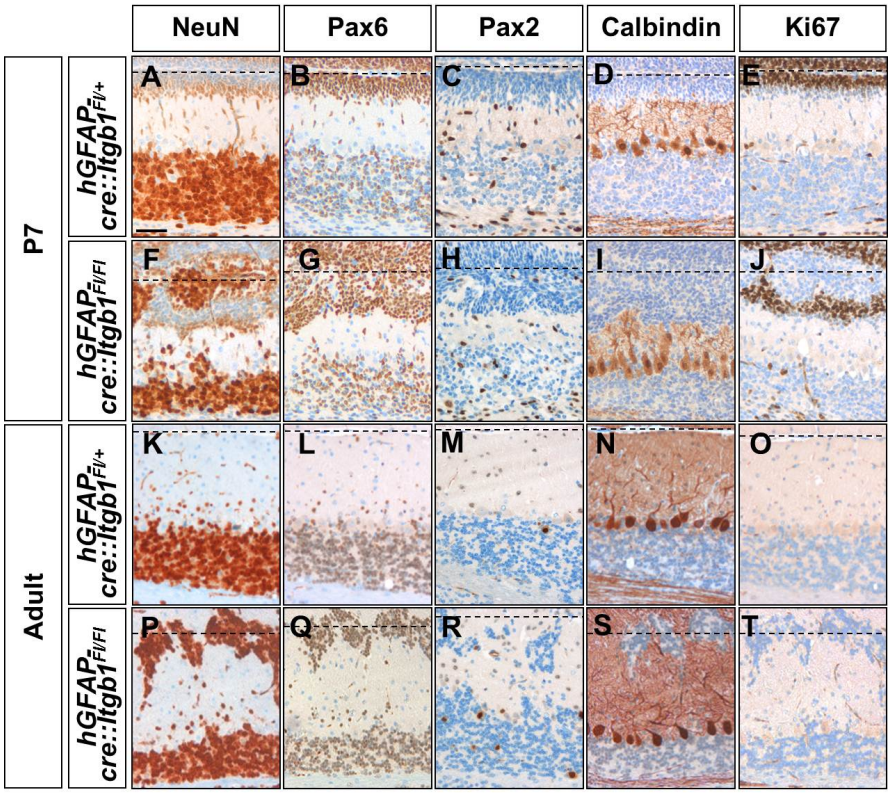

Figure 16 Analysis of different cell types in *hGFAP-cre::Itgb1*$^{Fl/Fl}$ mice compared with controls. Immunohistochemistry using indicated antibodies was performed on sections of paraffin-embedded cerebella from *hGFAP-cre::Itgb1*$^{Fl/+}$ (control) and *hGFAP-cre::Itgb1*$^{Fl/Fl}$ mice at postnatal day 7 (P7, A–J) and adult stage (K–T) to visualize the major cell types and proliferative activity in the cerebellar cortex. Dashed lines delineate the surface of the cerebellum between two cerebellar lobes. Scale bars: 50 µm (in A for A-T).

In this context, it was aimed to examine if the observed migration defect of cerebellar granule neurons in *hGFAP*cre*::Itgb1*$^{Fl/Fl}$ mice might be attributed alterations in the Bergmann glia cell population. To test this, the expression of different Bergmann glia markers was analyzed in the ML and the PCL. Whereas GFAP immunoreactivity in P7 cerebella was hardly detectable (Figure 17 A,E), the GFAP signal in adult *hGFAP*-cre*::Itgb1*$^{Fl/Fl}$ mice revealed that radial glial fibers extending from Bergmann glia showed no directional outgrowth to the cerebellar surface as compared with control mice and terminated randomly within the ML (Figure 17 I,M). The expression of Sox2 was restricted to Bergmann glia within the PCL in control mice according to the literature (Figure 17 B,J) (Sottile et al. 2006), but Sox2 expressing cells were found in the PCL,

the ML, and cerebellar granule neuron ectopia of *hGFAP*-cre*::Itgb1*$^{Fl/Fl}$ mice (Figure 17 F,N). To visualize Bergmann glia cells during postnatal development, the expression of S100 protein was also analyzed in the cerebellum of *hGFAP*-cre*::Itgb1*$^{Fl/Fl}$ mice. The vast majority of Bergmann glia cells inhabited the PCL in P7 and adult cerebella of control mice. Only few positive cells were detected in the ML or the IGL (Figure 17 C,K). In contrast, S100-positive cells invaded the ML and cerebellar granule neuron ectopia on the cerebellar surface of *hGFAP*cre*::Itgb1*$^{Fl/Fl}$ mice (Figure 17 G,O), further indicating a disorganization of the glial network. Last, antibodies against Cre were used that served as a surrogate marker of *hGFAP* in this system. Although Cre expressing cells in P7 control mice inhabited the PCL, the IGL, and the white matter (Figure 17 D), Cre expressing cells in P7 *hGFAP*-cre*::Itgb1*$^{Fl/Fl}$ mice were also detected in the ML, the EGL, or in ectopic cerebellar granule neuron accumulations (Figure 17 H). In the adult control cerebellar cortex, Cre immunoreactivity was only seen in Bergmann glia of the PCL (Figure 17 L), while in *hGFAP*-cre*::Itgb1*$^{Fl/Fl}$ mice Cre expressing cells were visible in the PCL and in the ML (Figure 17 P).

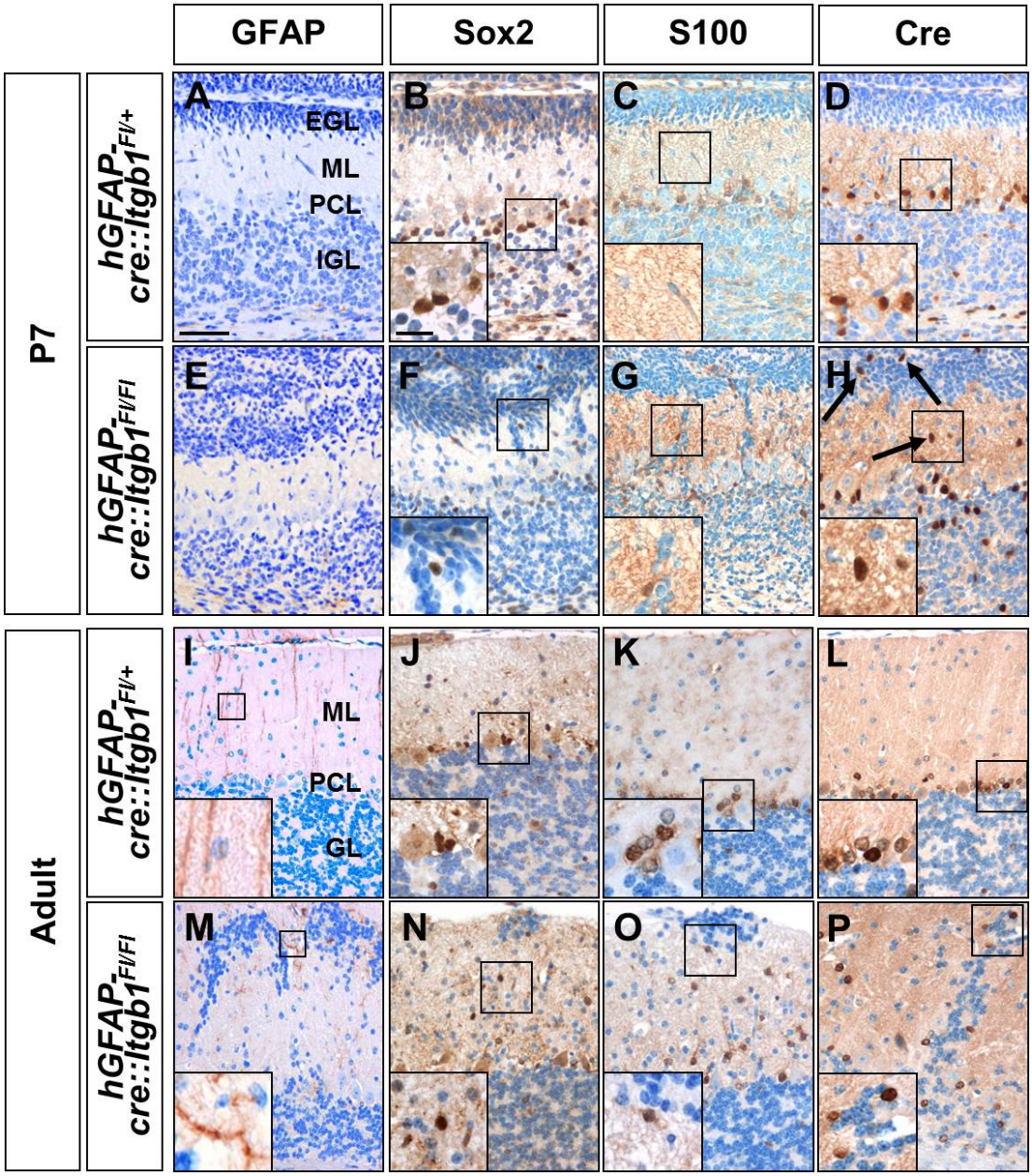

Figure 17 Translocation of Bergmann glia cells into the molecular layer in hGFAP-cre::Itgb1Fl/Fl mice. Cells positive for astrocytic markers hGFAP, Sox2, and S100 as well as Cre were predominantly localized in the Purkinje cell layer (PCL) of control mice, while a number of cells expressing these markers was distributed throughout the molecular layer (ML) in *hGFAP-cre::Itgb1*$^{Fl/Fl}$ mice both at postnatal day 7 as well as at adult stages (A–P). Scale bars: 25 µm (in A for A-P); 10 µm (inset in B; for all insets).

To further substantiate these findings, the number of cells was quantified in both the PCL and the ML that expressed S100 and Cre (Figure 18 A, B). The number of S100-positive cells in the PCL significantly decreased in *hGFAP*-cre*::Itgb1*$^{Fl/Fl}$ mice as compared with controls, while S100-positive cells in the ML markedly increased (Figure 18 A). At the same time, the overall number of S100-positive cells was not changed, indicating that Bergmann glia cells translocate from the PCL to the ML in *hGFAP*-cre*::Itgb1*$^{Fl/Fl}$ mice. These data are supported by quantification of Cre expressing cells in *hGFAP*cre*::Itgb1*$^{Fl/Fl}$ and control mice (Figure 18 B).

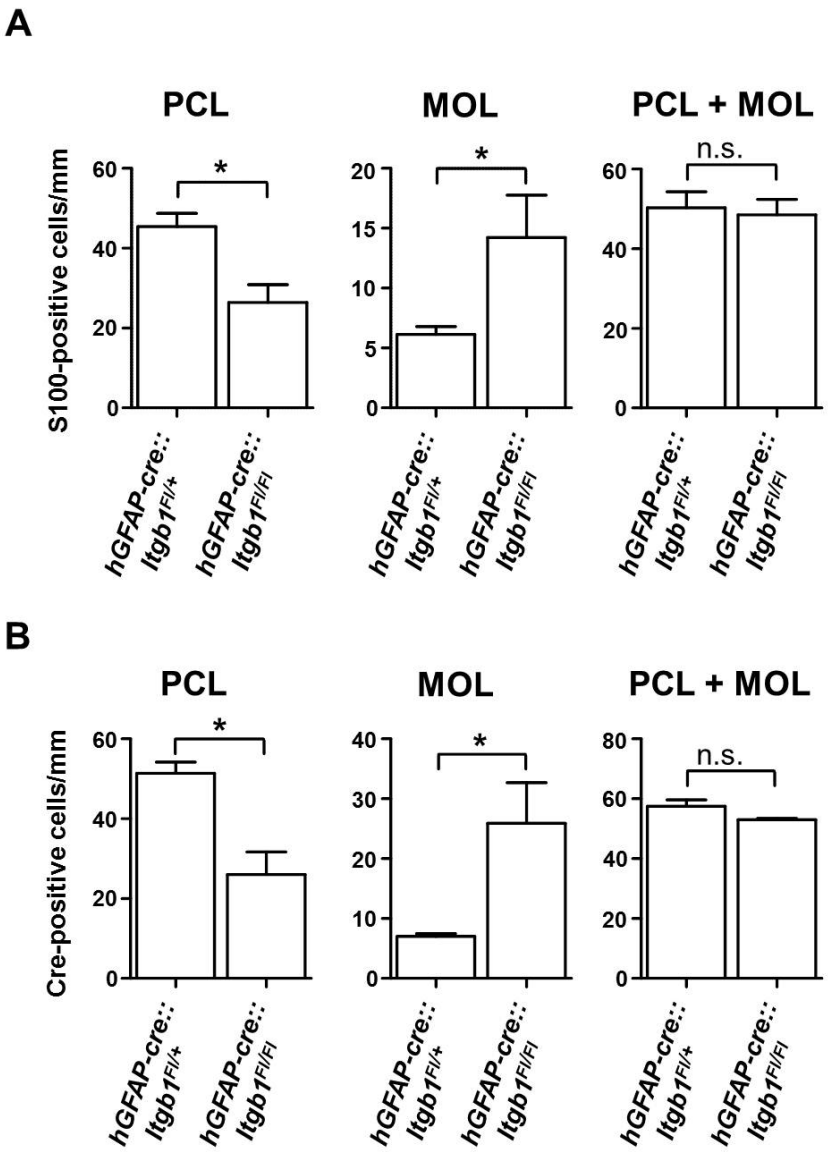

Figure 18 Quantification of S100- and Cre-positive cells in the PCL and ML at adult stage revealed that Bergmann glia cells translocate into the ML, while the overall number of Bergmann glia cells is not changed (A, B).

The conclusion is that loss of β1-Integrins in cerebellar glia has dramatic impact on the development of multiple cortical cell types. Although radial glia revealed pathological positioning and inappropriate development of its processes, cerebellar granule neurons were prohibited from physiological migration and underwent premature differentiation in ectopic regions of the superficial cerebellar cortex.

4. Discussion

In this study, β1-Integrin was inactivated selectively in granule neuron precursors of the cerebellum and in glia. Although the loss of β1-Integrins in glia has previously been described to result in reactive gliosis and in an activation of microglial cells in the forebrain (Robel et al., 2009), its role during cerebellar development was unclear, particularly with respect to glial-neuronal interactions. It was demonstrated that the loss of β1-Integrins in CGNPs did not affect the overall cerebellar development. In contrast, the deletion of β1-Integrins in glia resulted in severe malformations of the cerebellum, including the fusion of cerebellar folia and a disturbed migration of CGNPs. It should be mentioned that *hGFAP* promoter sequences do not provide a Bergmann glia-specific way to drive Cre expression and consecutively delete β1-Integrin. In fact, *hGFAP*-cre is similarly fate-mapping granule neurons and astrocytes of the cerebellum (Zhuo et al. 2001). Although, it cannot be totally ruled out the contributions of other affected cell types to the phenotype observed in *hGFAP*-cre:*Itgb1*$^{Fl/Fl}$ mice, the results of this study suggest that the deletion of β1-Integrins in Bergmann glia is responsible for the observed malformations for the following reasons: First, *Math1*-cre-driven β1-Integrin knockout in granule neurons alone did not result in any cerebellar pathology. Second, the observed migratory deficit of cerebellar granule neuron progenitors points to the Bergmann glia cells as the primary cause of the phenotype (Rakic and Sidman 1973). Third, massive morphological abnormalities were detected within the Bergmann glia cell population. The described impairment of cerebellar lamination in *hGFAP*-cre:*Itgb1*$^{Fl/Fl}$ mice is in line with previous findings that described the deletion of β1-Integrins in early neural precursor populations using the *Nestin* promoter (Graus et al., 2001). Here, it had been proposed that β1-Integrins in glial cells are required for the anchorage of glial endfeet to the meningeal basement membrane and the overall assembly of the basement membrane. Glial network perturbations near the basement membrane were also described to be responsible for cerebellar granule neuron ectopia (Graus et al., 2001). In contrast, it has been proposed that β1-Integrins act at least in part cell autonomously in CGNPs to positively regulate the synergistic effect of laminin on Shh-induced proliferation (Blaess et al., 2004). Although Blaess et al. used primary CGNPs cultures from *Nestin-cre:Itgb1*$^{Fl/Fl}$ mice and mainly tested the interaction of β1-Integrins and laminin in the context of Shh-induced proliferation, in this study conditional knockout mice with a specific *Itgb1* knockout in granule neuron precursors were investigated, but it was not possible to find any impact of β1-Integrin expression in granule neurons. In fact, the *in vivo* results suggest that β1-Integrin expression in *Math1*-positive cerebellar granule neurons, which represent the majority, if not all

granule neurons, is dispensable for the development of the cerebellum. To exclude compensatory mechanisms that could take place in vivo and possibly obscure essential roles of β1-Integrin, Pierfrancesco Pagella performed experiments, where he acutely deleted β1-Integrin in primary cultured *Itgb1$^{Fl/Fl}$* CGNPs by Cre/loxP-based recombination, but he did not detect any effects on Shh associated proliferation (Frick et al. 2012). The above mentioned results are supported by previous findings, which demonstrated that the deletion of β1-Integrins in migrating neurons is not sufficient to disturb the formation of cell layers in the cerebral cortex and the morphological differentiation of cortical neurons (Belvindrah et al., 2007). Furthermore, in vitro experiments on cerebellar CGNPs using inhibitory antibodies against β1-Integrins did not reveal any impact on BrdU-incorporation (Le Dreau et al. 2010). Instead, Daniel Grammel demonstrated in his *in vitro* experiments that the deletion of β1-Integrins from radial glia abolishes the poly-L-ornithine/laminin-mediated extension of glial fibers, which subsequently results in an impairment of CGNP development (Frick et al. 2012).
It appears possible that redundant receptor systems partly compensate the loss of β1-Integrins in vivo since the glial fiber system and neuronal migration in the cerebellum of *hGFAP-cre::Itgb1$^{Fl/Fl}$* mice was only partly disturbed. Nevertheless, the data of this study suggest that all development changes seen in *Nestin-cre:Itgb1$^{Fl/Fl}$* mice (Graus et al., 2001) are caused by the loss of β1-Integrins in glia, but not in cerebellar granule neurons. Interestingly, the size of *hGFAP-cre::Itgb1$^{Fl/Fl}$* cerebella was significantly smaller when compared with controls, a phenomenon that could point toward major defects in the proliferation of cerebellar granule neurons. However, it can be argued that these proliferation defects are not due to granule cell-autonomous functions of β1-Integrins and that any impairment in granule neuron proliferation may be a secondary phenomenon due to inappropriate migration along β1-Integrin-deleted radial glia. This hypothesis is supported by a previous report, where inappropriate development of Bergmann glia secondarily leads to a marked reduction of CGNP proliferation (Hoser et al. 2007). β1-Integrins have been studied intensively in various cancer types. Several reports indicate that β1-Integrins facilitate tumor progression in breast carcinoma (Chung et al. 2004; Friedrichs et al. 1995; Weaver et al. 1997; Wewer et al. 1997; Yao et al. 2007). Furthermore, β1-Integrins have been implicated in other cancer types such as prostate or colorectal cancer (Fujita et al. 1995; Rabinovitz et al. 1995), and have shown to serve as a prognostic marker in gastric and breast cancer (Xu et al. 2010; Yao et al. 2007). However, conflicting data attribute β1-Integrins a tumor suppressor function in breast cancer or tumor formation in the skin (Owens and Watt 2001; Zutter et al. 1995). This study demonstrates that deletion of β1-Integrin from tumors that arise in a mouse model of CGNP-derived medulloblastoma did not result in a significant

survival benefit. This observation is in line with the results from *Math1-cre::Itgb1*$^{Fl/Fl}$ mice, where β1-Integrins are dispensable for the proper development and proliferation of CGNPs. Furthermore, mRNA expression levels of β1-Integrin in human medulloblastoma samples did not predict patient outcome and thus appear not to have a prognostic value in medulloblastoma. Therefore, high expression of β1-Integrins in human medulloblastoma samples rather reflects overall genetic setup of cancer cells and similarities to immature, proliferating CGNPs than highlighting a special role of β1-Integrins in the formation of this tumor type. This situation is different from what has been shown for other tumors such as breast cancer, and it may also be different in gliomas that are frequent brain tumors, most likely arising from glial precursor cells. Here, it has been attributed a considerable role of α6β1-Integrin in tumor progression (Delamarre et al. 2009), and it is tempting to speculate whether loss of β1-Integrins may result in reduced growth of glioma or enhanced survival.

5. Summary

5.1. Summary

β1-class Integrins play essential roles both in developmental as well as in cancer biology. Particularly, a *Nestin*-driven deletion of β1-Integrin receptors results in severe abnormalities of brain development including a laminar disorganization of cerebellar granule neurons. However, since *Nestin* is expressed in all kinds of neural precursors, these data do not allow conclusions to be drawn about the role of β1-Integrins in distinct neuronal and glial cell types. By generating conditional knockout mice using granule neuron specific *Math1*-promoter sequences, it is shown that the expression of β1-Integrins in cerebellar granule neurons is dispensable for the development of the cerebellum. In another conditional knockout mouse model, the deletion of β1-Integrin in medulloblastomas that are derived from cerebellar granule neuron precursor (CGNP) did not result in a significant survival benefit. Last, the prognosis of the patient cannot be predicted by the expression of β1-Integrin in human medulloblastoma samples. However, a β1-Integrin knockout using *hGFAP*-promoter sequences led to cerebellar hypoplasia, misplacement of Bergmann glia in the molecular layer, undirected outgrowth of radial glial fibers, and cerebellar granule neuron ectopia. The conclusion of this data is that the expression of β1-Integrin in cerebellar granule neurons is not important during its development or the formation of medulloblastoma. In fact, it is the expression of β1-Integrin in glia that is crucial for the proper development of the cerebellar cortex.

5.2. Zusammenfassung

Integrine der Klasse β1 spielen sowohl in der Entwicklungs- als auch in der Tumorbiologie eine wichtige Rolle. Insbesondere eine *Nestin*-gesteuerte Entfernung von β1-Integrin-Rezeptoren führt zu schweren Abnormalitäten der Gehirnentwicklung, einschließlich einer laminären Fehlorganisation von zerebellären Körnerzellneuronen. Jedoch wird *Nestin* in allen Typen von neuralen Vorläuferzellen exprimiert, deshalb können aus diesen Daten keine Rückschlüsse auf die Rolle von β1-Integrinen in einzelnen neuronalen und glialen Zelltypen gezogen werden. Durch die Erzeugung konditioneller Knockout-Mäuse, unter der Verwendung einer für Körnerzellneuronen spezifischen Math1-Promotorsequenz, wird gezeigt, dass die Expression von β1-Integrin in zerebellären Körnerzellneuronen für die Entwicklung des Kleinhirns entbehrlich ist. In einem weiteren konditionellen Knockout Mausmodell führt die

Eliminierung von β1-Integrin in Medulloblastomen, die aus Vorläufern zerebellärer Körnerzellneuronen hervorgehen, zu keinem signifikanten Überlebensvorteil. Zuletzt, kann die Prognose des Patienten nicht durch die Expression von β1-Integrin in humanen Medulloblastomproben vorhergesagt werden. Ein Knockout von β1-Integrin unter Steuerung von hGFAP-Promotorsequenzen führt allerdings zur Kleinhirnhypoplasie, zur falschen Lage der Bergmann Gliazellen in der molekularen Schicht, zum ungerichteten Auswachsen von radialen Gliazellfasern und ektopisch liegenden zerebellären Körnerzellneuronen. Die Schlussfolgerung aus diesen Daten ist, dass die Expression von β1-Integrin in Körnerzellneuronen des Kleinhirns während dessen Entwicklung oder der Bildung von Medulloblastomen nicht essentiell ist. Vielmehr ist die Expression von β1-Integrin in Gliazellen entscheidend für die normale Entwicklung der Kleinhirnrinde.

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