Pathogenesis of SHH medulloblastoma in mice

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and

**Localization of SHH medulloblastoma in mice depends on the age at its initiation.**

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### Table of Contents

**Summary** .................................................................................................................................................. 1

**Zusammenfassung** .................................................................................................................................... 3

1 **Introduction** .......................................................................................................................................... 5
   1.1 Function and structure of the cerebellum .......................................................................................... 5
   1.2 Development of the cerebellum ....................................................................................................... 6
   1.3 Medulloblastoma .............................................................................................................................. 9
      1.3.1 Classification of medulloblastoma .......................................................................................... 9
      1.3.2 Symptoms, diagnostics and therapy of medulloblastoma ..................................................... 12
         1.3.2.1 Resistance to SMO inhibition ....................................................................................... 14
      1.3.3 Altered signalling pathways .................................................................................................... 15
         1.3.3.1 The Wnt pathway ........................................................................................................... 17
         1.3.3.2 The Sonic hedgehog pathway ......................................................................................... 18
         1.3.3.3 Interaction of Wnt and Shh signalling ............................................................................ 19
      1.3.4 Mouse models ......................................................................................................................... 20
   1.4 Aim of the study ................................................................................................................................. 23

2 **Materials and methods** ....................................................................................................................... 25
   2.1 Experimental animal studies ............................................................................................................ 25
      2.1.1 Mouse strains ........................................................................................................................... 25
         2.1.1.1 Conditional knockout mice .......................................................................................... 25
         2.1.1.2 Inducible knockout mice ............................................................................................... 26
      2.1.2 Tamoxifen induction and BrdU pulse ....................................................................................... 26
      2.1.3 Treatment of mice with LDE225 in vivo .................................................................................. 27
      2.1.4 DNA extraction and genotyping .............................................................................................. 27
      2.1.5 Primary cell culture ................................................................................................................. 29
         2.1.5.1 Cerebellar granule neuron precursor cells .................................................................... 29
         2.1.5.2 Tumor cells .................................................................................................................... 30
   2.2 Histology and immunohistochemistry .............................................................................................. 31
      2.2.1 BrdU staining ............................................................................................................................ 31
   2.3 Molecular biological methods .......................................................................................................... 33
      2.3.1 RNA extraction, cDNA synthesis and qRT-PCR ..................................................................... 33
      2.3.2 Production of retroviral particles ............................................................................................. 35
2.3.3 Colorimetric MTT-assay ................................................................. 35
2.3.4 Cell Lines .................................................................................. 36
2.3.5 Lithium chloride treatment in vitro .............................................. 37
2.4 Statistical analyses ........................................................................ 38

3 Results .............................................................................................. 39

3.1 Establishment and characterisation of mouse models for SHH medulloblastoma 39
  3.1.1 Mouse model with SMO mutation .............................................. 40
  3.1.2 Mouse model with SMO and PIK3CA mutations ......................... 41
  3.1.3 Mouse model with PTCH1 mutation ........................................... 44
  3.1.4 Mouse model with PTCH1 and PIK3CA mutations ....................... 44
  3.1.5 Mouse model with MYCN amplification ...................................... 47
  3.1.6 Mouse model with MYCN amplification and TP53 mutation ......... 48

3.2 Localization of SHH medulloblastoma in mice depends on the age at its initiation 51

3.3 Effects of Wnt/β-catenin signalling on Shh-dependent medulloblastoma formation ................................................................. 57
  3.3.1 In vitro treatment with the Wnt agonist lithium chloride ............... 61

3.4 Preclinical treatment of mouse models of SHH medulloblastoma with Hh inhibitors ................................................................................. 63
  3.4.1 LDE225 treatment ....................................................................... 64
    3.4.1.1 LDE225 treatment in Math1-creERT2::Isl-SmoM2F+/+ mice ........ 64
    3.4.1.2 LDE225 treatment in Math1-creERT2::Isl-SmoM2F+/+ Isl-Pik3caF+/+ mice 66
    3.4.1.3 LDE225 treatment in Math1-creERT2::Ptch1F+/F1 mice .......... 68

4 Discussion ........................................................................................ 70
  4.1 Localization of SHH medulloblastoma in mice ............................... 70
  4.2 Wnt/β-catenin signalling and Shh-dependent medulloblastoma ......... 72
  4.3 Establishment and characterization of mouse models ....................... 74
  4.4 LDE225 treatment of Shh-associated mouse models ....................... 76
  4.5 Perspectives .................................................................................. 81

5 References ....................................................................................... 84

6 Appendix .......................................................................................... 104
  6.1 Supplementary Figures ................................................................ 104
6.2  List of Figures .................................................................106
6.3  List of Tables ...............................................................108
6.4  List of Abbreviations ....................................................109
6.5  Gene names and definition of gene symbols ......................112
6.6  Curriculum Vitae ..........................................................114
6.7  List of publications .......................................................115
6.8  Acknowledgement ..........................................................116
Medulloblastoma (MB) is the most common malignant brain tumor of childhood that comprises at least four molecularly distinct subgroups. One subgroup is characterized by aberrant Sonic hedgehog (SHH) signalling. Despite the ubiquitous activation of the SHH pathway within this subgroup, there is clear evidence that tumors with a SHH profile may vary in certain molecular and clinical aspects. Targeted therapies as a novel treatment modality for MB patients are especially intriguing for primary and relapsed SHH-MB. As more drugs targeting the Hh pathway become available and enter clinical trials, it is important to know how to stratify the patients for different drugs in order to maximize response rates and to prevent unnecessary treatment failures.

In a first project, we aimed to characterize tumor localization in murine Shh-associated models. Using well established mouse models we show here that oncogenic transformation of cerebellar granule cell precursors at early developmental time points may result in the formation of midline and hemispheric medulloblastoma. On the other side, oncogenic transformation at later developmental time points exclusively results in the formation of hemispheric medulloblastoma. These data, which perfectly match to the recently published observations in human patients, indicate that granule neuron precursors are biologically distinct in different cerebellar compartments and that localization of SHH medulloblastoma is dependent on the time rather than the kind of genetic alteration.

In a second project, we aimed to investigate the effect of the Wnt pathway on Shh-associated medulloblastoma. Previous studies were able to show that Wnt/β-Catenin activation might be able to inhibit Shh-associated medulloblastoma growth through downregulation of the Shh pathway. Considering a possible therapeutic approach, in vitro treatments with lithium chloride, a well-known Wnt/β-Catenin agonist, were carried out. Lithium chloride treatment in vitro resulted in a decrease of granule neuron precursor and tumor cell viability.

In the third project, we aimed to better understand the mechanisms in MB of primary and secondary resistance to drugs targeting the Hh pathway. To this end, we generated new
mous models, characterized and used these and already existing genetically engineered mouse models (GEMMs). We aimed to further expand our pre-existing repertoire of murine SHH-activated MB models in order to have a spectrum of tumors that are driven by mutations at different levels of the Hh pathway, such as PTCH1, SMO, or MYCN, and with different combinations of other mutations in additional pathways that may cooperate with HH signalling (e.g. TP53 or PIK3CA). The mouse strains Math1-creERT2::Isl-SmoM2F1/+, Math1-creERT2::Isl-SmoM2F1/+ Isl-Pik3caF1/+, Math1-creERT2::Ptch1F1/F1, Math1-creERT2::Ptch1F1/F1 Isl-Pik3caF1/+, and Math1-creERT2::Ptch1F1/F1 Isl-Pik3caF1/F1 developed Shh-associated medulloblastoma. Our mouse model with a MYCN mutation did not develop any tumor, neither as such nor with an additional TP53 mutation. Math1-creERT2::Isl-SmoM2F1/+, Math1-creERT2::Isl-SmoM2F1/+ Isl-Pik3caF1/+, and Math1-creERT2::Ptch1F1/F1 mice were then treated with the SMO inhibitor LDE225, that is already used in clinical trials. Math1-creERT2::Isl-SmoM2F1/+ and Math1-creERT2::Ptch1F1/F1 mice first benefit from the treatment, but then also die due to symptoms of the tumor, whereas Math1-creERT2::Isl-SmoM2F1/+ Isl-Pik3caF1/+ mice did not show a better prognosis for survival at all. Nonetheless, proliferation was reduced in all tumors treated with LDE225.
Zusammenfassung

Das Medulloblastom (MB) ist ein maligner Hirntumor, welcher vornehmlich im Kindesalter auftritt und in der hinteren Schädelgrube mit starkem Bezug zum Kleinhirn entsteht. Basierend auf variierenden globalen Expressionsprofilen wird das humane Medulloblastom in vier verschiedene molekulare Subgruppen eingeteilt. Während eine dieser Subgruppen durch eine pathologische Aktivierung des Sonic hedgehog (SHH)-Signalwegs charakterisiert ist, weisen trotz dieser Gemeinsamkeit auch die Tumore innerhalb dieser Subgruppe ein beträchtliches Maß an Heterogenität bezüglich molekularer und klinischer Parameter auf. Gezielte Therapien als neuartige Behandlungsmethode für MB-Patienten sind besonders faszinierend für primäre und rezidivierende SHH-MB. Da mehr Medikamente verfügbar sind, die auf den HH-Signalweg abzielen, und in klinischen Studien eingehen, ist es wichtig zu wissen, wie man die Patienten für verschiedene Medikamente stratifiziert, um die Ansprechrate zu maximieren und unnötige Behandlungsfehler zu vermeiden.


In einem zweiten Teilprojekt sollte der Effekt des Wnt-Signalwegs auf Shh-assoziierte Medulloblastome untersucht werden. Frühere Studien konnten zeigen, dass die Wnt/ß-Catenin-Aktivierung in der Lage sein könnte das Wachstum von Shh-assoziierten Medulloblastomen durch die Herunterregulation des Shh-Signalweges zu hemmen. In

Im dritten Teilprojekt zielten wir darauf ab, die Mechanismen von Medulloblastomen mit primären und sekundären Resistzenzen gegenüber Substanzen, die gegen den Hh-Signalweg gerichtet sind, besser zu verstehen. Dazu wurden neue Mausmodelle erstellt und charakterisiert, und diese und bereits vorhandene gentechnisch hergestellte Mausmodelle (GEMMs) genutzt. Das bereits vorhandene Repertoire an murinen SHH-aktivierten MB-Modellen sollte weiter ausgebaut werden, um ein Spektrum von Tumoren zu erhalten, mit Mutationen, die auf verschiedenen Ebenen des HH-Signalweges, wie PTCH1, SMO oder MYCN basieren, und mit verschiedenen Kombinationen anderer Mutationen von zusätzlichen Signalwegen, die mit dem HH-Signalweg kooperieren (z.B. TP53 oder PIK3CA). Die Mauslinien Math1-creERT2::Isl-SmoM2Fl/+, Math1-creERT2::Isl-SmoM2Fl/+ Isl-Pik3caFl/+ Math1-creERT2::Ptcp1Fl/Ft, Math1-creERT2::Ptcp1Fl/Ft Isl-Pik3caFl/+ und Math1-creERT2::Ptcp1Fl/Ft Isl-Pik3caFl/+ entwickelten Shh-assoziierte Medulloblastome. Unser Mausmodell mit einer MYCN Mutation entwickelte keine Tumoren, auch nicht mit einer zusätzlichen TP53 Mutation. Math1-creERT2::Isl-SmoM2Fl/+ Math1-creERT2::Isl-SmoM2Fl/+ Isl-Pik3caFl/+ und Math1-creERT2::Ptcp1Fl/Ft Mäuse wurden anschließend mit dem Smo-Inhibitor LDE225 behandelt, der bereits in klinischen Studien genutzt wird. Math1-creERT2::Isl-SmoM2Fl/+ und Math1-creERT2::Ptcp1Fl/Ft Mäuse profitieren zuerst von der Behandlung, sterben aber letztendlich an Symptomen des Tumors, wohingegen Math1-creERT2::Isl-SmoM2Fl/+ Isl-Pik3caFl/+ Mäuse überhaupt keine bessere Überlebensprognose aufweisen. Trotzdem wurde die Proliferation in allen mit LDE225 behandelten Tumoren reduziert.
1 Introduction

1.1 Function and structure of the cerebellum

The cerebellum is part of the motoric system. It receives sensory information of the brain stem and the spinal cord. The cerebellum on its own is not able to initiate movements, but is responsible for coordination and fine adjustment of course of motions. In this context it is responsible for back-up motor apparatus of posture and movement, the motor activity and eye movement controlled of the cerebrum. In addition, it controls the muscle tone and maintains the balance.

The cerebellum lies in the posterior fossa dorsal to the brain stem (Mesencephalon, Pons, Medulla oblongata). Between the cerebellum and the brain stem lies the fourth ventricle filled with cerebrospinal fluid (CSF). The three cerebellar peduncles (Pedunculus cerebellaris superior, medius, inferior) connect the cerebellum with the brain stem and contain afferent as well as efferent fibres. The structuring of the cerebellum in the cerebellar vermis (Vermis cerebelli) and both lateral hemispheres (Hemispheria cerebelli) are clearly visible. The whole cerebellum contains a fine surface structure which consists of fissures (Fissurae cerebelli) and foliage (Folia cerebelli). As well as in the cerebrum (Telencephalon), the outward lying structure which is rich of neurons is the cortex (Cortex cerebelli) and the inner lying white substance with many fibres is the central white matter of the cerebellum (Corpus medullare cerebelli).

The adult cerebellar cortex is subdivided into three layers: the molecular layer (ML), the purkinje cell layer (PCL) and the inner granule cell layer (IGL) (Figure 1). Here the ML mainly consists of basket and stellar cells, the PCL consists of purkinje cells and Bergman glia cells and the IGL consist of mature granule cells, golgi cells, and astrocytes. The white matter with nerve cell fibres, astrocytes, and oligodendrocytes and the deep cerebellar nuclei complete the structure of the cerebellum (Altmann 1997).
1.2 Development of the cerebellum

The cells of the central nervous system (CNS) can be broadly divided into glial cells and neuronal cells, both differentiate from multipotential neural progenitor cells (Edlund and Jessell 1999).

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 dendritic arborisation (Palay and Chan-Palay 1974). The cell bodies of the Bergmann glia lie in the region of the cell bodies of Purkinje cells.
and their fibres pass the outermost molecular layer to the pial surface (Grosche et al. 2002, Welsch 2006). In addition to the Bergmann glial fibres also the processes of the cerebellar granule neurons, the parallel fibres, and the dendritic arborisation of the Purkinje cells extend into the molecular layer.

The development of the cerebellum in mice starts around embryonic day 9 (E9) with the development of the cerebellar anlage and finishes around postnatal day 20 (P20) (Hatten and Heintz 1995). Here the cerebellum descends from rhombomer r1, the rostral part of the rhombic lip, which is confined by the expression of the transcription factors Otx2 and Hoxa2 and is called the upper rhombic lip (Joyner 1996, Wingate and Hatten 1999, Joyner et al. 2000). The rhombomers r2-r8 form in their entirety the lower rhombic lip (Landsberg et al. 2005). In detail, all cells of the cerebellum stem from two different germinal epithelia. Purkinje cells, cells of the deep cerebellar nuclei and more than 6 different kinds of inhibitory interneurons like e.g. Golgi-, basket- and stellar- cells stem from the dorso-medial ventricular zone along the IV. ventricle (Hallonet and Le Douarin 1993, Dino et al. 2000, Hoshino et al. 2005). The second germinal epithelium, the rhombic lip, is the point of origin of the cerebellar granule cells as well as of a subpopulation of the deep cerebellar nuclei and neurons of different pre-cerebellar nuclei in the brain stem (Wingate and Hatten 1999, Wingate 2001, Machold and Fishell 2005, Wang et al. 2005).

In detail, the development of the cerebellum starts, as in all chordates, with neurulation, the development of the neural tube, as basis for the later central nervous system (CNS). Of the three embryonic germ layers (endoderm, mesoderm and ectoderm) only the central part of the ectoderm contributes to the development of the neural plate and the following folding of the neural tube. The unfolding happens at the neural plate by the development of the neural groove and the dorsal closing to a tubular structure, which represents the early spinal cord. In the inside of the neural tube is the Liquor cerebrospinalis, which holds the cavity open for the proper formation of the brain. The neurulation is induced by soluble growth factors of the notochord and the roof plate, which cause a compartmentalization of the embryonic CNS by dorso-ventral gradients of BMP-4 (bone morphogenetic protein 4) and SHH (sonic hedgehog) (Watanabe et al. 1998). The cerebellar primordium develops out of a gap in the dorsal neural tube, which leads to a lateral protrusion on the level of the pontine bend. The dorso-lateral regions of the wing plate bend medially and build up the rhombic lip (RL). Through growth
movement and protrusion of the rhombic lip to dorsal, a transversal placed plate is formed, the neural plate (Duband 2010). The rhombic lip is one of the both germinal zones of the developing cerebellum. Here granule cell precursors originate. Purkinje cells, as an example for cells of the ventricular zone, proliferate in their germinal zone only between embryonic day 11–14 (E11-E14). As these cells migrate radial in the developing cerebellum after they left the cell cycle, granule cell precursors migrate out of the upper rhombic lip, starting at E13–E15, tangential through the surface of the cerebellar anlage and there build up a secondary proliferation zone, the external granule cell layer (EGL) (Hatten and Heintz 1995, Hatten et al. 1997). The mitogen sonic hedgehog (Shh), which is generated by the purkinje cells, leads to a massive expansion of the granule cell population (Wechsler-Reya and Scott 1999). Here the EGL can be subdivided into a superficial zone of proliferating cells and an inner zone of differentiating cells. This subdivision is evident by the expression of different marker genes (RU-49, NeuN, etc.), which are differentially expressed during cerebellar development (Hatten and Heintz 1995, Weyer and Schilling 2003). The localization of these marker genes has shown that the development of cerebellar granule cells can be subdivided into at least 4 phases: neurogenesis, differentiation, migration and generation of synaptic connections. After they have left the cell cycle, granule cells migrate radially along the extensions of the Bergman glia cells into the cerebellum and there form the internal granule cell layer (IGL) (Rakic and Sidman 1973).
1.3 Medulloblastoma

Medulloblastoma (MB) is the most common malignant brain tumor in children. Current treatments for MB include surgical resection followed by irradiation of the entire neuraxis and high-dose chemotherapy. Many patients die despite these treatments, and those who survive often suffer from cognitive deficits and endocrine disorders as a consequence of therapy (Mulhern et al. 2005).

1.3.1 Classification of medulloblastoma

Since 2016 the World Health Organization (WHO) classifies MB based on histology and also molecular features (Louis et al. 2016). For histology several subtypes of the disease are recognized: classic, large cell/anaplastic (LCA), nodular/desmoplastic and MB with extensive nodularity (Louis et al. 2007). Patients with nodular/desmoplastic histology tend to have favourable outcomes, while those with large cell and anaplastic (LCA) histology have the worst prognosis (Eberhart et al. 2002, McManamy et al. 2007). Recent advances in microarray and genomic sequencing technologies have enabled a deeper understanding of MB. Based on such analysis, MBs have now been divided into 4 major molecular subgroups: WNT, Sonic Hedgehog (SHH), Group 3 and Group 4 (Jones et al. 2012, Northcott et al. 2012, Pugh et al. 2012, Robinson et al. 2012, Taylor et al. 2012). The WNT- and the SHH-subgroup are characterized by a constitutive activation of different, highly conserved signalling pathways (WNT- and SHH-signalling) (Figure 2). Since 2016, SHH-activated MB are subclassified into a TP53-mutant and a TP53-wildtype group (Louis et al. 2016).
The WNT subgroup shows a very good long-term prognosis in comparison to other subgroups (Ellison et al. 2005, Clifford et al. 2006, Rogers et al. 2009, Ellison et al. 2011). WNT-associated tumors, which occur in children and teenagers as well as in adults, normally have disrupted WNT signalling genes, including activating mutations in CTNNB1 (β-catenin), which activates canonical WNT signalling, and inactivating mutations in the adenomatous polyposis coli (APC) gene, a negative regulator of the WNT pathway (Hamilton et al. 1995, Zurawel et al. 1998). Other common features of the WNT subgroup are chromosome 6 loss and expression of the WNT-gene DKK1 (Thompson et al. 2006, Northcott et al. 2011). WNT MBs typically have classic histology (Rogers et al. 2009).

Analogous to the WNT subgroup, MBs of the SHH subtype show a characteristic activation of the Sonic hedgehog signalling pathway (Kool et al. 2008, Northcott et al. 2011, Schwalbe...
et al. 2011, Kool et al. 2012). Medulloblastoma of the SHH subtype (about 25% of all MBs) are the best analysed subgroup (Hatten and Roussel 2011). Common are somatic mutations in genes of this signalling pathway like inactivating mutations in the negative regulators PATCHED1 (PTCH1) or Suppressor of Fused (SUFU), activating mutations in the signal transducing molecule SMOOTHENED (SMO), but also amplifications of the SHH-target genes GLI1 and GLI2 transcription factor can lead to the constitutive activation of the pathway (Johnson et al. 1996, Hahn et al. 1999, Northcott et al. 2009, Adamson et al. 2010, Pfister et al. 2010, Northcott et al. 2011, Taylor et al. 2012). Many SHH-associated MBs have desmoplastic/nodular histology, although classic and LCA histologies are also observed (Taylor et al. 2012). SHH MB occurs in infants, where the prognosis is favourable, as well as in adults, where the prognosis is more variable (Kool et al. 2012). In this subgroup, there is a special age distribution. Mainly infants and adults develop SHH medulloblastoma, children quite rarely (Northcott et al. 2011). This biphasic age distribution presumes a huge heterogeneity in clinical, molecular and developmental biological parameter in this group. In addition, localization of human SHH MBs differs depending on the age of the patient. For so far unknown reasons, adult SHH MBs are almost exclusively located in cerebellar hemispheres, whereas infant SHH MBs often grow in the vermis (Wefers et al. 2014). It is known that an activation of the SHH pathway in granule cell precursors of the rhombic lip and the external granule cell layer (EGL) can lead to the development of Shh-associated Medulloblastoma (Schüller et al. 2008). Recently, it was shown that SHH-associated MB can also arise from granule neuron precursors of the cochlear nucleus of the brainstem (Grammel et al. 2012).

The majority of MBs do not exhibit activation of the WNT or SHH pathways, and these tumors can be divided into at least two subtypes – Group 3 and Group 4 – based on gene expression, DNA copy number changes and mutations. Group 3 MB patients commonly exhibit amplification or overexpression of the MYC oncogene and have gene signatures resembling those of photoreceptors and gamma-aminobutyric acid expressing (GABAergic) neurons (Taylor et al. 2012). In contrast, Group 4 tumors often exhibit amplification of CDK6 and MYCN or duplication of the Parkinson’s Disease-associated gene synuclein alpha interacting protein (SNCAIP), and have expression profiles reminiscent of glutamate-expressing (glutamatergic) neurons (Northcott et al. 2012).
These four subgroups are highly distinct in tumor histology and biology, and in addition show divergent clinical phenotypes such as differences in patient demographics, tumor dissemination, and patient outcome (Kool et al. 2012, Northcott et al. 2012, Taylor et al. 2012). Recently, a large series of pediatric MB was analysed using next generation sequencing technologies to map the genomic landscape of MB and to identify novel driver mutations in each molecular subgroup (Parsons et al. 2011, Jones et al. 2012, Northcott et al. 2012, Northcott et al. 2012, Pugh et al. 2012, Rausch et al. 2012, Robinson et al. 2012). As there are still many MB, for which a clear driver mutation is not identified yet, it was also analysed the epigenome of a large series of MB using whole genome bisulfite sequencing and ChIP-seq for various histone modifications to investigate, to what extent epigenetics plays a role in MB tumorigenesis and whether epigenetic alterations may explain the formation of tumors, in which a clear driver mutation was not found (point mutations, small insertions or deletions or focal copy-number aberrations) by WGS (Hovestadt et al. 2014).

1.3.2 Symptoms, diagnostics and therapy of medulloblastoma

First clinical symptoms are typically unspecific symptoms of an increased intracranial pressure: head ache, impaired vision, morning sickness and vomiting (Alston et al. 2003). Very young patients can develop a hydrocephalus due to the blockade of the circulation of the cerebrospinal fluid (CSF). This means that the increased fluid pressure in the ventricular system of the whole brain leads to the deformation of the still flexible calvaria. The primary localization of the medulloblastoma in the cerebellum causes the increased appearing ataxia in the course of the disease. Dizziness and problems with coordination as well as a general impairment of motor function are typical for progressive tumor growth. Neurological deficits, which are typical for the secondary affected brain structures can be observed at infiltrating growth and metastases. Diagnosis is made with imaging techniques (magnetic resonance imaging, MRI), where you can evaluate localization, size and extent of the tumor. For validation of the diagnosis and for therapeutic approach the dissected tumor tissue is examined by histology, immunohistochemistry and molecular methods. Independent of the subgroup, the treatment consists of a combination of resection, irradiation and chemotherapy (Evans et
al. 1990, Tait et al. 1990, Kortmann et al. 2000, Taylor et al. 2003). For very young patients you have to balance the chance of success of the irradiation and the psychomotor and cognitive disturbances in the early infantile development and the coincided postnatal development of the cerebellum (Duffner et al. 1993). Despite of substantial progress, actual therapy approaches still show unwanted secondary effects and hence resulting significant death rate. Due to the detailed characterisation of the subgroups of medulloblastoma and the analysis of the cell of origin of the different subtypes, therapies can be fitted to the proteins or cell populations involved. Due to the well-known influence of the SHH pathway on the development of medulloblastoma, many low-molecular connections were discovered, which can repress the activity of the SHH pathway as inhibitors of the transmembrane receptor Smoothened. The efficacy of these inhibitors could already be proven in allografts and mouse models for medulloblastoma (Berman et al. 2002, Romer et al. 2004, Romer and Curran 2005) and are analysed in clinical studies at the moment for the benefit in the human system (Coon et al. 2010). Molecular analyses of primary medulloblastoma promise much progress with regard to targeted cancer therapies, which have the molecular, cytological and developmental biological characteristics of the tumors as basis.

Targeted therapies as a novel treatment modality for MB patients are especially intriguing for primary and relapsed SHH-MB. As more drugs targeting the Hh pathway become available and enter clinical trials, it is important to know how to stratify the patients for different drugs in order to maximize response rates and to prevent unnecessary treatment failures. Current clinical trials targeting SHH-MB with SMO antagonists (such as LDE225) use a five-gene signature to identify SHH-MB. However, this signature is not sufficient as a predictive biomarker for response to SMO antagonists, since it detects all SHH-MB with Hh activation regardless of their underlying genetic makeup (Kool et al. 2014). Indeed, it is clear from clinical trials using SMO antagonists that the response appears to be highly variable with a large proportion of striking durable responders, but also with a lot of non-responders. Recent data have shown that SHH-MB are genetically very heterogeneous and the response to drugs antagonizing the Hh pathway strongly depends on the type of mutation that activates the pathway. To understand the mechanism(s) of primary resistance and to identify pathways co-operating with aberrant Hh signalling, a large cohort of SHH-MB (n = 133) was sequenced and profiled. Hh
pathway mutations identified in this study involved *PTCH1* (found across all age groups), *SUFU* (mainly found in infants, including many in the germline), and *SMO* (mainly in adults) (Figure 3).

![Figure 3 Hh pathway mutations in 133 sequenced SHH-MB. Cases have been split up in infants, children and adults, and are sorted based on the type of mutation in the Hh pathway. Results show that SHH-MB are genetically heterogeneous and the different age groups harbor different predominant mutations activating the Hh pathway (reprinted from with permission of Elsevier (Kool et al. 2014), copyright (2014), license number 3967660794571).](image)

Older children harbored an excess of downstream *MYCN* and *GLI2* amplifications and frequent *TPS3* mutations, often in the germline, all of which were exceedingly rare in infants and adults.

### 1.3.2.1 Resistance to SMO inhibition

Functional assays in different patient-derived SHH-MB xenograft models demonstrated that SHH-MB harboring a *PTCH1* mutation were responsive to SMO inhibition, whereas tumors harboring *SUFU* mutations or *MYCN* amplifications were primarily resistant. SHH-MBs with alterations in downstream SHH pathway genes, however, such as *SUFU, GLI2*, or *MYCN*, demonstrated primary resistance to SMO inhibition (Lee et al. 2007). These data showed for the first time that most adults, but only half of the pediatric patients with SHH-MB will likely be responsive to SMO inhibition as predicted by molecular analysis of the primary tumor and tested in the SHH-xenografts. SHH-MB patients resistant to SMO inhibitors need to be treated with other inhibitors targeting the pathway, for instance, at
the level of GLI. Furthermore, as has been shown in both humans and mice, tumors may also rapidly acquire secondary resistance to treatment (Rudin et al. 2009, Yauch et al. 2009, Dijkstra et al. 2011), suggesting that such inhibitors might be ineffective as a curative option when administered as monotherapy. It is strongly advocated that the next generation of SMO inhibitor trials should be based on underlying tumor genetics because many patients with SHH-MB will not respond to these inhibitors. Alternative treatment options could include arsenic trioxide (ATO) targeting GLI transcription factors by degrading the protein (Kim et al. 2013). ATO and the antifungal agent itraconazole (which acts on SMO) have also been suggested in preclinical experiments for use in SHH-MBs that become resistant after treatment with SMO antagonists (Kim et al. 2013) or in combination with SMO inhibitors upfront knowing that \textit{GLI2} amplifications comprise a common mechanism of secondary resistance to SMO inhibition in preclinical models (Buonomici et al. 2010, Dijkstra et al. 2011). Recurrent mutations in additional pathways also suggested rational combination therapies including epigenetic modifiers and PI3K/AKT inhibitors, especially in adults. Other options for combination therapies to avoid or delay the development of resistance include drugs targeting PI3K/AKT/mTOR- or PKA-signalling pathways, both mutated in a subset of patients with SHH and both also leading to GLI activation (Milenkovic and Scott 2010, Wang et al. 2012, Metcalfe et al. 2013), or epigenetic drugs. Most MB molecular data have been generated for primary resected tumors, but patients usually die of relapses/metastases that do not respond anymore to therapy. Therefore, it is of utmost importance to gain insight in the molecular alterations that are present in these relapses and/or metastases to improve the survival of these relapsed patients.

1.3.3 Altered signalling pathways

The dysregulation of two evolutionary highly conserved signalling pathways can lead to disturbances of postnatal development of the cerebellar cortex and to the development of medulloblastoma. These pathways are the WNT- and the Sonic hedgehog (SHH)- pathway (Figure 4).
Introduction

Figure 4 The WNT and SHH pathway. These two major signalling pathways regulate a wide range of developmental events, particularly the proliferation of cerebellar granule cells by sonic hedgehog (SHH). Both pathways have been implicated as targets for disruption in childhood solid tumors (CSTs), including medulloblastoma, a granule-cell-derived brain tumor. WNT and SHH bind to the cell-surface receptors frizzled (FRZ) and patched (PTCH), respectively. Binding of WNT activates its receptor, which then blocks the phosphorylation-activated (by glycogen synthase kinase-3ß (GSK3ß) in a complex with APC and axin) degradation of ß-catenin (ß-Cat). As a consequence, ß-catenin translocates to the nucleus and activates transcriptional events that, in some contexts, promote proliferation. Binding of SHH inhibits PTCH function and so releases smoothened (SMO) to activate intracellular events. SMO represses the cleavage of GLI (the cleaved form has a transcriptional-repressor role in the nucleus), resulting in its release from a complex including fused (FU) and costal (COS). GLI is subsequently translocated to the nucleus and, as a result, activates target genes that, in some contexts, produce a pro-proliferative response. So, there is a great deal of similarity between these two pathways. Furthermore, suppressor of fused (SUFU) represses both pathways by promoting nuclear export of both ß-catenin and GLI. SUFU can also directly repress the transcriptional activity of intact GLI. Mutations in SUFU, ß-catenin, PTCH and SMO have all been implicated as causative events in medulloblastoma. Black steps are activating and red steps are inhibitory. Large arrows indicate movements within the cell, whereas thin arrows represent activating molecular targets. Thin T-bars indicate inhibiting molecular targets. DSH, dishevelled; TCF, T-cell factor; GRO, groucho. (reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Scotting et al. 2005), copyright (2005), license number 3967661323110)
1.3.3.1 The Wnt pathway

The Wnt (Wingless related integration site) pathway is a highly conserved signalling pathway, which was first discovered due to its role in carcinogenesis (Nusse and Varmus 1982) and body axis formation during embryonic development (Klaus and Birchmeier 2008).

In the inactive state of the WNT signalling pathway, $\beta$-catenin, which is coded by the gene $CTNNB$, is phosphorylated in the cytoplasm of the cell (Amit et al. 2002). $\beta$-catenin is ubiquitinated by a protein complex of APC, Axin, Ck1$\alpha$ and kinase GSK-3$\beta$ and thus degraded by the proteasomal route of degradation (Aberle et al. 1997, Behrens et al. 1998, Itoh et al. 1998). After binding of a WNT-agonist to the transmembrane receptor Frizzled, an intracellular signalling cascade leads to the inactivation of the protein kinase GSK-3$\beta$ whereby $\beta$-catenin is not phosphorylated anymore (Bhanot et al. 1996, Salic et al. 2000). Hence $\beta$-catenin can’t be ubiquitinated anymore and therefore is not degraded. In consequence, $\beta$-catenin reaches the nucleus where it functions as primary effector of the WNT-signalling and leads to the transcription of target genes as $Dkk1$, $MYC$, $Axin2$ and $CD44$ (He et al. 1998, Wielenga et al. 1999, Jho et al. 2002, Chamorro et al. 2005). The WNT pathway is pathologically altered in 10-15% of all medulloblastoma cases which led to the name of the WNT subgroup due to the characteristic activation (Roussel and Hatten 2011).

First indications for the involvement of this pathway in the development of medulloblastoma were made due to the fact that patients with the Turcot syndrome, which is caused by mutations in the tumor suppressor gene $APC$, tend to develop colon carcinoma and neuroepithelial brain tumors including medulloblastoma (Hamilton et al. 1995, Marino 2005). In addition, sporadic mutations in the $CTNNB1$ gene can often be found in this subgroup, which lead to stabilization of $\beta$-catenin und therefore to the activation of the WNT pathway (Zurawel et al. 1998).
1.3.3.2 The Sonic hedgehog pathway

In the inactive state of the hedgehog pathway, the transmembrane receptor Patched (PTCH1) inhibits the G-protein coupled receptor Smoothened (SMO) which leads to the inhibition of the whole signalling pathway. Ligands for the Patched receptor in mammals are three different proteins (sonic hedgehog, indian hedgehog, desert hedgehog) (Echelard et al. 1993). Through binding of SHH-ligands to the extracellular domain of the Patched receptor, the inhibition of Smoothened by Patched is removed and the signal is transmitted. Therefore Smoothened migrates into the primary cilium of the cell and causes the dissociation of the protein complex consisting of SUFU (Suppressor of fused homolog) and the transcription factors Gli2 and Gli3 (Corbit et al. 2005, Huangfu and Anderson 2006, Wang et al. 2009). After dissociation of SUFU, the transcriptions factors move into the nucleus and there regulate the gene expression (Aza-Blanc et al. 1997, Bai et al. 2002). The Sonic hedgehog pathway is significantly involved in the development of the cerebellum. During the postnatal development of the cerebellum, granule cell precursors move transversally out of the upper rhombic lip over the surface of the cerebellum and there build the external granular layer (EGL) (Altmann 1997). Sonic hedgehog, which is produced by Purkinje cells serves as growth factor for these granule cell precursors and leads to a rapid expansion of this cell population during the postnatal development (Hatten 1999, Wechsler-Reya and Scott 1999). In this context it is not surprising that mutations in genes of this signalling pathway which is important for the proliferation of granule cell precursors were early associated with the development of medulloblastoma. Thus, in patients with a germline mutation in the SHH-receptor PTCH1 the Gorlin-syndrome is manifested which is featured among other things by a predisposition for medulloblastoma (Bale et al. 1998, Taylor et al. 2000). In a similar way, germline mutations in the SHH-inhibitor SUFU also lead to an increased development of medulloblastoma (Taylor et al. 2002, Pastorino et al. 2009, Brugieres et al. 2010). Somatic mutations in genes of the SHH pathway like PTCH, SMO and SUFU, but also amplifications of the SHH target genes GLI1 and GLI2, were identified in sporadic medulloblastoma and are said to be the reason for the constitutive activation of this signalling pathway (Johnson et al. 1996, Hahn et al. 1999, Northcott et al. 2009, Adamson et al. 2010, Northcott et al. 2011). This spectrum of mutations together with the fact that a subgroup of medulloblastoma show a pathological activation of the SHH pathway indicates that a
dysregulation of this pathway plays an important role in the development of medulloblastoma. Furthermore, dysregulation of the Shh pathway plays a role in the induction of tumors occurring in different human tissues, such as Basal cell carcinoma (Athar et al. 2014) or Pancreatic ductal carcinoma (Lauth and Toftgard 2011). Molecules targeting different components of the Sonic hedgehog pathway, such as the Smoothened inhibitor Vismodegib, have already been subject of clinical trials aiming at the consolidation of novel therapeutic approaches against Shh-driven neoplastic pathologies (Sekulic et al. 2012).

In addition to the molecular distinctions among MB subtypes, a number of signalling pathways are found to be activated across multiple subtypes of the disease. For example, the phosphatidylinositol 3'-kinase (PI3K) pathway is activated in WNT (Robinson et al. 2012), SHH (Northcott et al. 2012), and Group 3 MB (Pei et al. 2012), and genes responsible for histone methylation and chromatin remodelling (MLL2, MLL3, KDM6A, EZH2, ZMYM3) are deregulated in both Group 3 and Group 4 MBs (Pugh et al. 2012, Robinson et al. 2012).

1.3.3.3 Interaction of Wnt and Shh signalling

It has been reported that sonic hedgehog (SHH) signalling and Wnt signalling interact in tumors. For example, in colorectal cancer, overexpression of Gli1 (a downstream component of the SHH signalling pathway) inhibits Wnt signalling and colorectal cancer cell proliferation, even in cells possessing the stabilizing mutation of β-catenin (Akiyoshi et al. 2006). In neural progenitors, Wnt signalling and SHH signalling coordinately regulate cell cycle progression, with SHH signalling activation required upstream (Alvarez-Medina et al. 2009). The Wnt/β-catenin and the Hedgehog (Hh) pathway interact in various cell types while eliciting opposing or synergistic cellular effects. Both pathways are known as exclusive drivers of two distinct molecular subtypes of MB. In sonic hedgehog (Shh)-driven MB, activation of Wnt signalling has been shown to suppress tumor growth by either β-catenin-dependent or -independent inhibition of Shh signalling (Anne et al. 2013, Peng et al. 2013, Pöschl et al. 2014). However, neither mechanistic insight into β-catenin-mediated inhibition of the Hh pathway, nor the
therapeutic potential of Wnt/β-catenin-activating drugs has been examined specifically in MB. Recently, Zinke et al. propose that beta-catenin stabilization increases its physical interaction with Gli1, leading to Gli1 degradation and inhibition of Hh signalling, thereby promoting tumor cell senescence and suppression of “tumor take” in mice (Zinke et al. 2015).

1.3.4 Mouse models

In order to better understand and analyse genetic, molecular and cytological influences on the development of medulloblastoma, different mouse models are available, which are based on different mutations and genetic systems depending on the group of tumor. It is currently thought that tumor cells of the SHH type of medulloblastoma originate from the granule cell progenitors in the external granular layer. Indeed the vast majority of these cells express markers of the proliferating external granular layer, the most common of which is ATOH1 (MATH1). More recently, it was demonstrated that Atoh1 protein expression was crucial for the progression of this type of tumor (Flora et al. 2009) because this transcription factor potently regulates the proliferation of granule cell progenitors in mice (Klisch et al. 2011). This likely occurs by Atoh1 maintaining the sensitivity of progenitor cells to Shh signalling through transcriptional activation of the Gli2 gene. Moreover, several groups have generated mouse models for key elements of the SHH subgroup of human medulloblastomas.

One of the first and most used genetically engineered mouse model (GEMM) for the Shh-associated medulloblastoma is based on a conventional knockout of the Shh-repressor Patched (Ptch1). This first Ptch1−/− mouse model was generated by homologous recombination in embryonic stem cells, in which part of Ptch exon 1 (including the putative start codon) and all of exon 2 were replaced with lacZ and a neomycin resistance gene (Goodrich et al. 1997). Homozygous deletion of Ptch1 (Ptch1−/−) in the mouse is embryonic lethal, but 20% of mice with a heterozygous Ptch1-knockout (Ptch1+/−) develop tumors in the cerebellum, which are molecularly very similar to human medulloblastoma (Goodrich et al. 1997, Hahn et al. 2000). Simultaneous loss of the tumor suppressor p53 (Tp53−/−) leads to a tumor incidence of 100% and illustrates cooperative functional

Activation of other components of the Shh pathway can also lead to the development of medulloblastoma. It has been shown via RCAS/TVA-systems that Nestin-positive precursor cells can develop medulloblastoma in 10-15% of cases after transduction with RCAS-based retrovirus which are coding for Shh (Rao et al. 2003, Broderick et al. 2004, Browd et al. 2006, Binning et al. 2008). Here RCAS (replication competent ASLV long terminal repeat with Splice acceptor)-based retrovirus can just transduce cells which express TVA (avian retrovirus receptor) under the Nestin promoter and therefore enables a cell-specific activation of the Shh pathway.

A very robust mouse model for Shh-associated medulloblastoma was generated by expression of a constitutive activated form of the *Smoothened* gene (SmoA1) under the granule cell precursor specific promoter ND2 (Hallahan et al. 2004, Hatton et al. 2008). Almost 100% of the animals developed a tumor which also metastasizes in the leptomeningeal region, a characteristic of many human tumors. Analogous to RCAS-based mouse model mentioned above, tumor development outside the CNS can be avoided.

At the moment the most popular mouse models for Shh-associated medulloblastoma are based on conditional systems like the Cre/loxP-recombination system, which allows a tissue- and cell type-specific recombination and therefore manipulation. Independent of the temporal activity of the promoter, mutations are activated cell type specifically by injection of an oestrogen analogue (Tamoxifen) (Leone et al. 2003, Erdmann et al. 2007, Arnold et al. 2011). The cell type specific activation of the Shh pathway with special Cre mouse strains (*Math1*-cre for granule cell precursors, *hGFAP*-cre for neural precursors) increased the tumor penetrance up to 100% without a simultaneous knockout of further tumor suppressors. Therefore the Shh-repressor *Ptch1* is promoter specifically deleted or a constitutive activated form of *Smoothened* gene (SmoM2-YFP) is transcribed, what is leading, in both cases, to an activation of Shh signalling and a subsequent development of medulloblastoma (Mao et al. 2006, Schüller et al. 2008, Yang et al. 2008).
While all mouse models mentioned above imitate human SHH-associated medulloblastoma, the number of mouse models for WNT-, Group 3- and Group 4-subgroups are very limited. Recently a mouse model for human WNT-associated medulloblastoma was described (Gibson et al. 2010). Here tumors were generated through activation of the Wnt pathway in cell populations of the dorsal brainstem with a simultaneous knockout of the tumor suppressor p53, which are molecularly similar to human WNT-medulloblastoma. For the first time it was shown that the cell of origin of WNT medulloblastoma lies not necessarily in the cerebellum. Interestingly, the comparison of the model for Shh- and Wnt-associated medulloblastoma showed that they really develop from different cell of origins (Gibson et al. 2010). Shh-medulloblastoma develop from the upper part of the rhombic lip, or in the auditory part of the lower rhombic lip, whereas Wnt-tumors develop in the precerebellar part of the lower rhombic lip (Grammel et al. 2012).

Limited data from two different mouse models for Group 3 medulloblastoma were generated through expression of c-MYC in cerebellar precursor cells and an additional knockout of p53 (Kawauchi et al. 2012, Pei et al. 2012). The fact that Group 3 medulloblastoma are the most aggressive subgroup with the least survival rate, illustrates the need to generate appropriate mouse models for this subgroup with regard to preclinical studies.

For Group 4 medulloblastoma no reliable mouse model exists at the moment.
1.4 Aim of the study

My focus of research is the development, diagnosis and therapy of medulloblastoma, the most malignant brain tumor in children. Medulloblastoma possibly develop very early, already during embryonic development.

Recent clinical trials for SHH-MB using SMO antagonists showed highly variable clinical responses including a large proportion of striking durable responders, but also a lot of non-responders. Recent sequencing data from an unprecedented series of SHH-MB (n=133) showed that about half of pediatric SHH-MB are predicted to be primarily resistant to these drugs because of mutations downstream of SMO. Moreover, it is known that some initially responsive tumors may also rapidly acquire secondary resistance to treatment, whereas other patients keep in remission for several years on a single targeted drug.

One part of this study deals with the localization of SHH MBs. Since we know that the localization of human SHH MBs differs depending on the age of the patient, we wanted to analyse this statement more precisely at different ages in an already existing mouse model for SHH MBs. The aim is to determine if such murine SHH MBs develop at special time points and/or at defined localizations.

Another point of this study intends to investigate the diverging role of synchronistic Wnt and Shh activation and deals with the question whether the physiological roles of Wnt/\(\beta\)-catenin signalling can be utilized to treat Shh-medulloblastomas. In vitro experiments were carried out, aiming at the possible use of Lithium, a Wnt activator, in the therapy of Shh-driven medulloblastoma.

Finally, we aimed at a better understanding of the mechanisms in MB of primary and secondary resistance to various drugs targeting the hedgehog pathway. To this end, we generated, characterized and used genetically engineered mouse models (GEMMs) of SHH-MB. We aimed to further expand our pre-existing repertoire of murine SHH-activated MB models in order to have a spectrum of tumors that are driven by mutations at different levels of the Hh pathway, such as in \(PTCH1\), \(SMO\) or \(MYCN\), and with different combinations of other mutations in additional pathway that may co-operate with Hh
signalling (e.g. TP53 or PIK3CA). We treated some of these models with drugs targeting the Hh pathway. We tested LDE225, a SMO inhibitor that already have entered clinical trials. Mice were monitored for treatment response, development of secondary drug resistance, and overall survival. Tumors will be molecularly characterized by sequencing and gene expression profiling, and data will be compared to untreated tumors.
2 Materials and methods

2.1 Experimental animal studies

2.1.1 Mouse strains

In this work wild type mice of the strains C57/Bl6 and FVB and a number of transgenic mouse models were used. SmoM2-YFP<sup>Fl/Fl</sup> (Mao et al. 2006), aiTdTomeato (Madisen et al. (2010), P<sub>t</sub>ch<sup>1-</sup>/ (Goodrich et al. 1997, Oliver et al. 2005), Ctnnb1(ex3)<sup>Fl/Fl</sup> (Harada et al., 1999; Pöschl et al., 2013), GSK3β<sup>Fl/Fl</sup> (Jaworski et al, 2011), Math1-GFP (Lumpkin et al., 2003), P<sub>t</sub>ch<sup>1-Fl/Fl</sup> (Uhmann et al., 2007), Pik3ca<sup>H1047R</sup> (Adams et al., 2011), P53 (Marino et al., 2000), Isl-Mycn (Althoff et al., 2015).

In addition, the driver lines Math1-cre (Matei et al., 2005; Schüller et al., 2007) and Math1-creER<sup>T2</sup> (Machold and Fishell, 2005) were used.

Mice were hold in a SPF-animal facility (specific-pathogen-free) in individually ventilated cages (IVC) with a constant light-dark rhythm of 12/12 hours. Food and water were given ad libitum. For preparation of the brain adult mice were sacrificed by cervical dislocation. Animals under 10 days were sacrificed by decapitation. For removal of the brain the cranial bone was opened and removed until the surface was laid open from the olfactory bulb anterior till the hindbrain. The brain was lifted out of the skull by cutting through the optical nerve at the optic chiasm and the brain nerves under the brain stem.

All experimental procedures were approved by the Government of Upper Bavaria, Germany (Reference number 55.2-1-54-2532-10-14 and 55.2-1-55-2532-56-15).

2.1.1.1 Conditional knockout mice

By mating a cre-driverline with one or more transgenic mouse strains, conditional Cre-loxP-systems are created. This means that the cre-recombinase is expressed under the promoter (here Math1-cre), which is able to initiate recombination between so called lox-P-Sites on the DNA and therefore delete everything located between the lox-P-Sites.
2.1.1.2 Inducible knockout mice

By induction with the oestrogen analogue tamoxifen mutations can be created under the Math1-promoter ($Math1$-creER$^{T2}$) at defined time points. Mice with this genetic background express the transgene after successful recombination promoter-specific. Under the Math1-promoter a transgenic oestrogen receptor is expressed which allows the translocation of the cre-recombinase from the cytoplasm into the nucleus only after binding of tamoxifen. Here the enzyme can remove the STOP-sequences in front of the genes by recombination. Thus the expression of the transgene depends on the activity of the Math1-promoter and the time point of the tamoxifen induction.

2.1.2 Tamoxifen induction and BrdU pulse

The tamoxifen-inducible mouse model $Math1$-creER$^{T2}$ makes it possible to activate the cre recombinase specifically during the postnatal development in Math1-positive granule cell precursors of the cerebellum. The fusion protein of the cre recombinase and a mutated form of the human oestrogen receptor is expressed under the Math1-promoter in this mouse line, but it is retained in the cytoplasm of the cell. Only after tamoxifen induction it reaches the nucleus and became active (Feil et al., 1997; Helms et al., 2000; Machold and Fishell, 2005). Mice with this inducible cre system were induced by tamoxifen (Sigma Aldrich) either at postnatal day 3 or 5 or pregnant dams were induced at embryonic day 14.5. Therefore 1 mg tamoxifen (in cornoil) is administered intraperitoneal (i.p.).

For analysis of the proliferation rate of cerebellar granule cells in vivo in different mouse models a bromodesoxyuridine (BrdU)–pulse was performed and mice were sacrificed 2 hours later. Therefore the animals were administered 25 µg/g body weight intraperitoneal. BrdU is integrated in the DNA as a chemical analogue of the nucleoside thymidine and can be verified immunohistochemically afterwards. After a 2 hour BrdU-pulse, cells which were in the S-phase of the cell cycle can be verified.
2.1.3 Treatment of mice with LDE225 \textit{in vivo}

Mice \(\text{Math1creER}^{T2::lsl-SmoM2^{Fl/+}}, \text{Math1-creER}^{T2::lsl-SmoM2^{Fl/+}} \text{Isl-Pik3ca}^{Fl/+} \text{and Math1creER}^{T2::Ptch1^{Fl/Fl}}\) were treated with the hedgehog inhibitor (via smoothened antagonism) LDE225, also known as Sonidegib/Erismodegib (sponsored by Novartis; marketed as Odomzo). Treatment start was at postnatal day 30 or 20. Each mouse was treated 5 times per week for three weeks long with a dose of 30 mg/kg/day (i.p.). LDE225 was dissolved in DMSO and this solution was then mixed with 40% polyethylene glycol (PEG). Each injection was 100 µl i.p. (5 µl LDE225 dissolved in DMSO + 95 µl 40% PEG were mixed directly before usage). The vehicle group was treated with 5 µl DMSO + 95 µl 40% PEG.

2.1.4 DNA extraction and genotyping

DNA for genotype verification through PCR and electrophoresis was extracted from mouse tail (early postnatal stages) or ear biopsies (older than 3 weeks). Each biopsy was first treated with 500 µl Laird’s lyses buffer (200 mM NaCl, 100 mM Tris buffer (pH 8.5), 5 mM EDTA, 0.2% SDS) and 10 µl Proteinase K (10 mg/ml) on a thermo-shaker at 56°C for at least 2 hours. Samples were then centrifuged (14,000 U) at room temperature for 5 minutes. The supernatant containing the DNA was then extracted into a new tube and 500 µl Isopropanol were added. After mixing, the precipitated DNA became visible. Centrifugation at room temperature with 14,000 U for 5 minutes was repeated. Finally, the remaining Isopropanol was discarded and the DNA pellet was resuspended in TE-Buffer (20 nM Tris-HCl pH 8.3, 1 mM EDTA in ddH₂O) and then stored at 4°C.

The genomic DNA (gDNA) is the starting material for genotyping. Therefore genotype-specific regions of the genome were amplified by polymerase chain reaction (PCR). Primers are listed in Table 1 according to primary descriptions of the mouse strains in original publications.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5’ – 3’</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre-Fw</td>
<td>TCCGGGCTGCCACGACCAA</td>
<td>448 bp</td>
</tr>
<tr>
<td>Cre-Rv</td>
<td>GGCGCCGGCAACACCCATTATT</td>
<td></td>
</tr>
<tr>
<td>SmoM2-YFP Mut-Fw</td>
<td>GAACGGGATCAAGGTGAAA</td>
<td></td>
</tr>
<tr>
<td>SmoM2-YFP Mut-Rv</td>
<td>CGATGGGGGTGGTTCTGCT</td>
<td>109 bp</td>
</tr>
<tr>
<td>SmoM2-YFP WT-Fw</td>
<td>GGAGCGGGAGAAATGGATATG</td>
<td></td>
</tr>
<tr>
<td>SmoM2-YFP WT-Rv</td>
<td>CGTGAATCTGCAAATCCAGTC</td>
<td>410 bp</td>
</tr>
<tr>
<td>tdTomato Mu-Fw</td>
<td>GGCATTAAGGACAGGTATCC</td>
<td></td>
</tr>
<tr>
<td>tdTomato Mu-Rv</td>
<td>CTGTTCTCTGACGCAAGTGG</td>
<td>196 bp</td>
</tr>
<tr>
<td>tdTomato WT-Fw</td>
<td>AAGGGAGCTCGAGTGGAAGT</td>
<td></td>
</tr>
<tr>
<td>tdTomato WT-Rv</td>
<td>CCGAATATCTGGAGGAGGTC</td>
<td>297 bp</td>
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<td>Ctnnb1(ex3) Fw</td>
<td>CGTGGCAATGGCTACTCAA</td>
<td>330 bp (wildtype),</td>
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<tr>
<td>Ctnnb1(ex3) Rv</td>
<td>TGTTCAACTTGGTATCCAG</td>
<td>500 bp (mutant)</td>
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<td>GSK3β Fw</td>
<td>TCTGGGCTTACTAGTCTAG</td>
<td>302 bp (wildtype),</td>
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<tr>
<td>GSK3β Rv</td>
<td>CAAAGTTATGGAAATGGGA</td>
<td>488 bp (mutant)</td>
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<td>Math1 GFP WT Rv</td>
<td>GAAGGGCATTTGGTGTCTCAG</td>
<td>314 bp (mutant)</td>
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<td>MYCN MU Fw</td>
<td>ACCACAAGGCGCCCTAGTACC</td>
<td></td>
</tr>
<tr>
<td>MYCN MU Rev</td>
<td>TGGAAGGACAGTGTAGG</td>
<td>168 bp</td>
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<tr>
<td>MYCN WT Fw</td>
<td>CTCTTCCCCTGATCTGCAAATCC</td>
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<td>MYCN WT Rev</td>
<td>CATGTCTTTTAACTACTCTCAGATGG</td>
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<td>CACAAAAAACAGGTTAACCAGGC</td>
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<td>P53 Rv</td>
<td>GCAACCTTTGATCCCAGCAGATA</td>
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<td>TTCAATGGACACTTTGGGGAAC</td>
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<td>AGTGGTGAGACACAGATCAGC</td>
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<td>TTTACTGGCTCGTTTTAACAACGTGTA</td>
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<td>AAAGTGCTCTGAGTTTGTAT</td>
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<td>Pik3ca 2</td>
<td>GCGAGAGGTGTTCCTCAACC</td>
<td>310 (mutant),</td>
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<td>Pik3ca 3</td>
<td>GGAGGCGGGAGAAATGGGATAG</td>
<td>410 bp (Pi3k)</td>
</tr>
</tbody>
</table>

Table 1 List of primers for genotyping.
Standard conditions the genotyping PCR are as follows (Table 2):

<table>
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<tr>
<th>step</th>
<th>temperature [°C]</th>
<th>time [s]</th>
</tr>
</thead>
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<td>95</td>
<td>120</td>
</tr>
<tr>
<td>denaturation</td>
<td>95</td>
<td>30</td>
</tr>
<tr>
<td>primer hybridisation</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35 cycles</td>
</tr>
<tr>
<td>elongation</td>
<td>72</td>
<td>60</td>
</tr>
<tr>
<td>stop</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

Table 2 PCR conditions for genotyping.

Analysis of the amplified DNA-fragments was carried out by 1% agarose gels and the Syngene gel documentation system (Synoptics Limited).

2.1.5 Primary cell culture

2.1.5.1 Cerebellar granule neuron precursor cells

Well plates were coated with 1x poly-L-ornithine (PLO) (Sigma) from a 100x stock (10 mg/ml). Dilution was carried out in H₂O and 0.2 µM sterile filters were used in order to preserve poly-L-ornithine. The plates were then incubated at 37°C during mouse preparation allowing poly-ornithine to polymerise. Mouse cerebella were dissected at postnatal day 5-7 and put on ice in 15 ml Hanks buffered saline solution (Gibco, pH 7.4 Glucose 6 mg/L). After centrifugation at 4°C for 5 minutes with 800 rpm, HBSS was discarded. 1 ml of 1x Trypsin/EDTA/DNase (100 µg DNase/ml, both Trypsin/EDTA and DNase were from Sigma) was added and the cerebella were left 10 minutes in water bath for incubation. Trypsin was then inactivated with 2 ml of DMEM-F12 + 10% fetal bovine serum (fetal bovine serum was inactivated at 56°C for 30 minutes prior to usage). Next, the cerebella were centrifuged at 4°C with 1500 g for 5 minutes. The fetal bovine serum medium was aspirated and substituted with 5 ml HBSS solution. The pellets were dissolved by pipetting gently and a new centrifugation step (as above) was conducted. After discarding supernatant HBSS, the pellets were resuspended in 1 ml HBSS and spun
as above. The HBSS remnant was extracted and the cells were put into suspension with culture medium with supplements (DMEM-F12, 1% N2 Supplement, 1% Penicillin/Streptomycin, 0.25 mM KCl with 10% FCS. All reagents were from Invitrogen). The poly-L-ornithine solution was removed and each well was washed with PBS once. Granule neuron precursors were then plated in serum-free culture medium with supplements at a concentration of 1 million cells/well and grown at 37°C and 5% CO₂. After 6-12 hours the medium was removed and culture medium with Shh-protein (3 µg/ml) was added. Due to culturing with this medium for at least 24 hours, all cells except cerebellar granule cells leave the cell cycle and a homogeneous proliferating granule cell population remained. For transduction of granule cells with IRES-GFP and Cre-IRES-GFP virus, the medium was removed and the virus supernatant (see 2.5.3 production of retroviral particles) was added to the cells for 4 hours. Due to the fact that retrovirus just transduce proliferating cells and culturing in selective Shh-medium, it was prevented that other cell types than granule cells could be transduced with the virus. After virus transduction cells were again cultured in culture medium with Shh-protein for 24 hours. For analysis of the proliferation rate bromodesoxyuridin (BrdU, 25 µg/ml) was added to the medium, and cells were fixed after two hours with 4% paraformaldehyde.

2.1.5.2 Tumor cells

Tumor cell culture was performed as described for culturing GNPs with the following exceptions: tumor cells were maintained in Neurobasal medium containing B27 supplement, human FGF (20 ng/ml), mouse EGF (20 ng/ml), glutamin (2 mM) and Pen/Strep (100 µg/ml). Here no Shh-protein was added to the medium because cultured tumor cells from our mouse strains for Shh-associated medulloblastoma have a constitutive activated Shh signalling pathway anyway.
2.2 Histology and immunohistochemistry

For all histological methods, brains were dissected, fixed overnight in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 µm according to standard protocols. Paraffin sections were deparaffinised with xylene and rehydrated in a graded alcohol series. In general morphology was analysed with Hematoxylin and eosin (H&E) stains. H&E stains were performed according to standard protocols.

All histological photomicrographs were taken digitally using an Olympus BX50 microscope in combination with the ColorView (Soft Imaging System).

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. For inactivation of the endogene peroxidase all slides were incubated in 5% H₂O₂ in methanol. Then the slides were washed in PBS, blocked for 30 min in I-Block reagent (Invitrogen) and incubated over night with the primary antibody at 4°C.

All primary antibodies for immunohistochemistry and immunocytochemistry are listed in Table 3.

<table>
<thead>
<tr>
<th>antibody</th>
<th>species</th>
<th>Catalog #</th>
<th>vendor/provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU</td>
<td>mouse</td>
<td>10198</td>
<td>Bioscience Products</td>
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<tr>
<td>BrdU</td>
<td>rat</td>
<td>ab6326</td>
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</tr>
<tr>
<td>GFP</td>
<td>rabbit</td>
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<tr>
<td>RFP</td>
<td>rabbit</td>
<td>AA234</td>
<td>Antibodies online</td>
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<tr>
<td>Sox2</td>
<td>rabbit</td>
<td>ab97959</td>
<td>Abcam</td>
</tr>
</tbody>
</table>

Table 3 Primary antibodies for immunohistochemistry and immunocytochemistry.

Staining was performed using the HRP/DAB staining system (DAKO) according to the manufacturer’s specifications. For immunofluorescence staining slides were washed with
Materials and methods

PBS after incubation with the first antibody and incubated with a species-specific, fluorophore-coupled secondary antibody (Alexa 488 and Alexa 546, Invitrogen). Staining of the nuclei was done by 4',6-Diamidino-2-phenylindol (DAPI; Roth). For fluorescent staining of cell culture the same protocol was used as for immunofluorescence staining, but without deparaffination and rehydration. Pictures of fluorescent staining were taken with an Olympus IX50 microscope.

2.2.1 BrdU staining

The immunohistochemical detection of the incorporation of the base analogue BrdU in the DNA instead of the physiological nucleoside thymidine requires another pretreatment before the incubation with the first antibody. To permit the access for the BrdU-specific antibody to the antigen, DNA has to be denaturated with 2N HCl for 10 min and then the acid has to be neutralized with 0.1 M sodium borat buffer (pH 8.5), not to impair the structure of the primary antibody.
2.3 Molecular biological methods

2.3.1 RNA extraction, cDNA synthesis and qRT-PCR

Both cultured cells and tissue samples were first put into Eppendorf tubes and homogenised with 750 µl TRlzol® (Invitrogen) reagent using a pipette, before proceeding with RNA extraction. The homogenised samples were let sit at room temperature for about 5 min. 150 µl of chloroform were added to each tube. After mixing for 15 sec, the samples were incubated at room temperature for 3 min. A centrifugation step with 14,000 rpm at 4°C was conducted for 15 min, after which the supernatant, RNA-containing top layer was extracted into a new Eppendorf tube. The RNA was then precipitated with cold (4°C) isopropanol and incubated at room temperature for 10 min. A centrifugation step was repeated as described above. Eventually, the RNA pellet became visible at the bottom of the tubes. The supernatant liquid was discarded and the RNA pellet was washed with 75% ethanol. After brief shaking, each sample was centrifuged for 5 min at 4°C with 7500 rpm. Ethanol was discarded, and the RNA pellets were resuspended in 24 µl DEPC water each. The RNA concentration was determined through Nanodrop 3300 technology (Thermo Fischer Scientific). cDNA synthesis for RT-PCR was performed using SuperScript® III First-Strand Synthesis Supermix (Invitrogen). Each component was mixed and centrifuged before use. For each RNA sample the reaction mix consisted of the following reagents: 6 µl of RNA (in DEPC water, up to 5 µg RNA), 0.5 µl oligo(dT)20 primer (50 µM), 0.5 µl of random hexamers (50 ng/µl), 1 µl annealing buffer, RNAse/DNAse-free water to bring the total volume up to 8 µl. After heating at 65°C for 5 min in a thermocycler, the tubes were put on ice for 1 min. Leaving the tubes on ice, the following solutions were added to each reaction mix: 10 µl First-Strand Reaction Mix (2x), 2 µl SuperScript® III/ RNaseOUT™ Enzyme Mix. The tubes were than mixed by vortexing and centrifuged briefly. The samples were incubated in a thermocycler for 5 min at 25°C, 50 min at 50°C and finally at 85°C for 5 min. Finally, the cDNA was cooled down on ice and stored at -20°C.

Quantitative real-time PCR (qRT-PCR) was carried out using the LightCycler® 480 (Roche) technology and the LightCycler® 480 SYBR Green I Master Mix (Roche). SYBR Green is a fluorescent cDNA-intercalating dye, which is detected and quantified after each elongation step, therefore allowing cDNA quantification in real time. Each reaction mix
Materials and methods

contained the following agents: FastStart Taq DNA Polymerase and SYBR Green dye (Roche), 3 µl of cDNA, 1 µl forward primer and 1 µl reverse primer (primer stocks concentration: 100 pmol/µl). Before usage, each primer was diluted 1:10 in DNAse free water. A RT-PCR program was conducted, which included the following steps: heat shock at 95°C for 5 min, then 45-cycles containing the following steps: denaturation at 95°C for 10 seconds, annealing at 57-62°C for 10 seconds, elongation at 72°C for 15 seconds. Relative quantification was achieved by comparing cDNA levels between target genes and housekeeper genes (β2-Microglobulin (β2M)). Besides this, a calibrating sample was used for each LightCycler® run within a single experiment, in order to compensate variations of the reaction mix composition. All samples were analysed in triplicates. The following formula was applied for relative quantification:

\[ NR = E_T^{CpT(C) - CpT(S)} \times E_R^{CpR(S) - CpR(C)} \]

NR=normalized ratio, E=primer efficiency, T=target gene, C=calibrating sample, R=housekeeper gene, S=sample, Cp=crossing point (number of cycles after which the SYBR Green detection threshold is reached).

All primers were designed using the Primer3 software and ordered from Eurofins Genomics. Amplicon melting curves were analysed to determine the presence of a single PCR product, primer quality and primer efficiencies were calculated based on standard curves. Table 4 shows primer pairs sequences and efficiencies.

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<th>gene</th>
<th>sequence</th>
<th>efficiency</th>
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<tr>
<td>β2M Rv</td>
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<td>Gli1 Fw</td>
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<td>60°C</td>
</tr>
<tr>
<td>Gli2 Rv</td>
<td>GCTGGCCGTCCCAACTGCTT</td>
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<td></td>
</tr>
</tbody>
</table>

Table 4 Primers for qRT-PCR.

Standard conditions for RT-PCR are summarized in Table 5.
### Materials and methods

<table>
<thead>
<tr>
<th>step</th>
<th>detection mode</th>
<th>temperature [°C]</th>
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<td>denaturation</td>
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<td>95</td>
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<td>4</td>
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</tr>
</tbody>
</table>

Table 5 Conditions for qRT-PCR.

#### 2.3.2 Production of retroviral particles

Retroviral particle for transduction of primary mouse cells were produced by transfection of human HEK293T cells (*American Type Culture Collection*, ATCC). The work with eukaryotic cells was done with sterile vessels and solutions in a sterile environment (HERAsafe, Thermo). HEK293T cells were cultured in incubators at 37°C and 5% CO₂. For production of retroviral particles, HEK239T cells were transfected in a triple-transfection with two helper plasmids (pCMV-VsVg and pCMV-gag-pol) and each with a retroviral construct with only IRES (internal ribosome entry-site)-GFP or with a Cre (Cre-recombinase)-IRES-GFP sequence using the transfection reagent X-tremeGENE HP (Roche). The medium was replaced after 24 hours with normal culture medium (DMEM medium, 10% foetal calf serum, 1% Glutamax). The supernatant of the retroviral medium was taken up to 4 days after transfection and cleaned with 0.45 mm filters and stored at -80°C.

#### 2.3.3 Colorimetric MTT-assay

The colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-assay is a spectrophotometer-based method used to quantify cell growth or cytotoxicity rates (Finlay et al. 1986, Vistica et al. 1991). The tetrazolium salt MTT is chemically
Materials and methods

reduced by ribosomes in metabolic active cells, forming formazan crystals. The resulting change of solution colour is then quantified through spectrophotometric absorbance measure. For this work, the Cell Proliferation Kit I (MTT) from Roche was used. Cells were first seeded in 100 µl in 96-well plates at a concentration of 2x10⁴/well and incubated (37°C) in neurobasal culture medium with supplements (see also 2.1.5 Primary cell culture) for 16 hours. Spectrophotometric absorbance was determined using the FLUOstar Optima (BMG labtech) system. For lithium chloride treatment, start point of therapy was defined by adding 10 µl of the MTT reagent to at least three wells and adding the Solubilisation solution (100 µl) after 4 hours, in order to quantify cell viability at time point 0 (T₀). After 24 hours (T₁), 10 µl of the MTT reagent were put into the remaining wells (at least in triplicates) for 4 hours, after which, the Solubilisation solution (100 µl) was added into each well. One well was left without MTT and served as a blank sample. The plates were let sit in the incubator overnight and the optical density (OD) at T₀ and T₁ was determined as follows: ODₜ₀ – ODblank. Cell viability after 24 hours (T₁) was calculated using the following formula: ODₜ₀/ODₜ₁ = 100%/x.

2.3.4 Cell Lines

HEK 293T cells arise from human embryonic kidney cells, which were originally transformed with Adenovirus 5 DNA (Graham et al. 1977). UW473 are a human medulloblastoma cell line obtained from a pediatric tumor sample (Bobola et al. 2005, Castro-Gamero et al. 2013). GL261 cells derive from the GL261 mouse glioma model. This murine model was first developed through application of a chemical carcinogen into the brain of C57BL/6 mice and then perpetuated by means of syngeneic transplantation (Maes et al. 2011). All three cell lines proliferated as adherent monolayers and were cultured in DMEM medium with 10% fetal calf serum, 1% Glutamax (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen) and 1% HEPES buffer (Sigma).
2.3.5 Lithium chloride treatment *in vitro*

In our *in vitro* setting, lithium chloride was balanced with NaCl in order to achieve equal salt concentrations of 25 mM in every well.
2.4 Statistical analyses

The Prism5.02 software (GraphPad) was used for statistical analysis. Survival data were obtained through Kaplan-Meier curves and the Log-rank test served as significance test. P-values < 0.05 were considered as significant. When comparing two groups with assumed Gaussian distribution and equal variances, the unpaired t-test was conducted. In case of not equal variances or an expected non-Gaussian distribution, the non-parametric Mann-Whitney test was used. For testing distributions for their significance between two groups, the $\chi^2$ test was used. For RT-PCR and MTT-assays a number of at least three samples was analysed for each experimental condition or mouse genotype.
3 Results

3.1 Establishment and characterisation of mouse models for SHH medulloblastoma

Components of the canonical Hh signalling pathway are Hh ligands such as sonic hedgehog (SHH), the transmembrane protein PTCH1 that acts as a receptor for Hh ligands, SMO, a transmembrane protein and signalling partner of PTCH1, and the family of GLI transcription factors (Zibat et al. 2010). In the absence of SHH, PTCH1 inhibits SMO. Binding of SHH to PTCH1 suspends this PTCH1-mediated inhibition of SMO, which in turn activates GLI transcription factors, resulting in the expression of target genes including GLI1. GLI1 activity is increased upon its phosphorylation by e.g. PI3K/AKT/mTOR (Figure 5).
Recent clinical trials for SHH-MB using SMO antagonists showed highly variable clinical responses including a large proportion of striking durable responders, but also a lot of non-responders. Recent sequencing data (Kool et al. 2014) from an unprecedented series of SHH-MB (n = 133) showed that about half of pediatric SHH-MB are predicted to be primarily resistant to these drugs because of mutations downstream of SMO. Moreover, it is known that some initially responsive tumors may also rapidly acquire secondary resistance to treatment, whereas other patients keep in remission for several years on a single targeted drug.

In order to establish novel therapies that are based on the individual genetic and molecular profile of a patient’s SHH-MB, we wanted to expand our battery of genetically engineered mouse models (GEMMs) based on mutations at different levels of the Hh signalling cascade. Apart from using already established mice with mutations in PTCH1 and SMO we wanted to generate novel additional models that faithfully model human SHH-MB with amplifications of e.g. MYCN. All these models are also be modified with respect to additional mutations in TP53 or PIK3CA, strictly representing the findings in human SHH-MB.

All mice were exposed to tamoxifen and monitored for symptoms of tumor growth, head growth, drinking behaviour, movements, weight and neurological symptoms, such as ataxia, paralysis or seizures. Animals that developed symptoms were sacrificed and one half of the cerebella were subjected to histological analysis to confirm the presence of tumors, and of the other half of the cerebella tumor tissue was harvested for further analysis.

### 3.1.1 Mouse model with SMO mutation

Here we used the Cre-LoxP-recombination system to ensure the constitutive activation of the Shh pathway. Therefore the enzyme cre-recombinase is able to cut out so called floxed sequences in the genome of mice, which are framed by loxP-sequences in vivo. For the constitutive activation of the Shh pathway a floxed mouse strain was available which allows a cre-mediated, conditional expression of a mutated Smo-allele (SmoM2-YFP) together with YFP-sequences (Mao et al. 2006). Mice with a mutation in the Smoothened
receptor \((\text{SmoM2})\) under the tamoxifen-inducible \(\text{Math1-creERT}^{\text{T2}}\)-promoter (MATH1 is a marker for cerebellar granule precursor cells) were generated. Through administration of tamoxifen at postnatal day 3 (P3), the Shh pathway can be constitutively activated in cerebellar granule cell precursors which leads to the development of SHH-associated medulloblastoma and often to the development of a hydrocephalus. The tumor incidence is 100%. Here the heterozygosity (one activated \(\text{SmoM2}\) allele) is sufficient for tumor development \((\text{Math1-creERT}^{\text{T2}}::\text{Isl-SmoM2}^{\text{Fl/+}})\). The median survival of these mice is 65 days (Figure 6 i). Tumor mice developed symptoms like abnormal head growth, ataxia and loss of weight. All tumors display classic histology (Supplementary Figure 25).

### 3.1.2 Mouse model with \(\text{SMO}\) and \(\text{PIK3CA}\) mutations

The phosphoinositide 3-kinase (PI3K) signalling pathway is one of the most frequently mutated pathways in cancer and plays an important role for proliferation in human tumors. The \(\text{Pik3ca}^{\text{H1047R}}\) model was first described by (Adams et al. 2011). H1047R mutations in the kinase domain account for approximately 40% of breast cancer \(\text{PIK3CA}\) mutant alleles (Saal et al. 2005). Although PIK3CA mutations are common in adult cancers (Samuels et al. 2004) and reported in medulloblastoma (Broderick et al. 2004), their role in tumorigenesis remains controversial. In particular it is not known if these mutations initiate or progress cancer.

\(\text{Math1-creERT}^{\text{T2}}::\text{Pik3ca}^{\text{Fl/+}}\) and \(\text{Math1-creERT}^{\text{T2}}::\text{Pik3ca}^{\text{Fl/Fl}}\) mice were induced at P3 and analysed. No abnormalities in brain development, no tumor development or abnormalities in survival could be observed.

Analogous to the previous mouse model, mice with an additional constitutive activated form of \(\text{PIK3CA}\) were generated. Tumor incidence after tamoxifen administration was 100%. \(\text{Math1-creERT}^{\text{T2}}::\text{Isl-SmoM2}^{\text{Fl/+}}\ \text{Isl-Pik3ca}^{\text{Fl/+}}\) mice showed a faster tumor development and first symptoms (e.g. head growth or ataxia) occurred earlier compared to \(\text{Math1-creERT}^{\text{T2}}::\text{Isl-SmoM2}^{\text{Fl/+}}\) mice (Figure 6). Kaplan-Meier analysis of these two mouse models show that mice with both (\(\text{SMO}\) and \(\text{PIK3CA}\)) mutations have a significantly \((p < 0.0001)\) worse survival rate than mice with only a \(\text{SMO}\) mutation. The median survival of \(\text{Math1-creERT}^{\text{T2}}::\text{Isl-SmoM2}^{\text{Fl/+}}\ \text{Isl-Pik3ca}^{\text{Fl/+}}\) mice is 48 days. All tumors display classic
Results

histology (Supplementary Figure 25). MBs in this mouse strain often have contact to the brainstem (13/18) (e.g. Figure 6 j, k).

Here, heterozygous as well as homozygous PIK3CA mutations alone are not enough to generate a tumor in our mouse model, but leads to a faster tumor progression in Math1-creERT2::Isl-SmoM2Fl/+ mice.

Figure 6 (a-l) shows an overview of Math1-creERT2::Isl-SmoM2Fl/+, Math1-creERT2::Pik3caFl/+, Math1-creERT2::Pik3caFl/Fl and Math1-creERT2::Isl-SmoM2Fl/+ Isl-Pik3caFl/+ mice: sagittal section H&E and proliferation (Ki67).
Figure 6 Overview Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/\textasciitilde}, Math1-creER\textsuperscript{T2}::Isl-Pik3ca\textsuperscript{Fl/\textasciitilde}, Math1-creER\textsuperscript{T2}::Isl-Pik3ca\textsuperscript{Fl/Fl} and Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/\textasciitilde} Isl-Pik3ca\textsuperscript{Fl/\textasciitilde} mice. Mice were induced with Tamoxifen at P3 and sacrificed.
Results

at P30. Left column (a, d, g, j) shows sagittal H&E stains of the whole brain (scale bar 1 mm), middle column (b, e, h, k) shows proliferation (Ki67 stains) of the cerebellum (scale bar 500 µm); Scale bar is 10 µm for higher magnifications (c, f, I, l). The Kaplan-Meier analysis (m) shows a sig. lower survival rate for Math1-creERT2::Isl-SmoM2Fli/+ lsl-Pik3ca Fli/+ mice (n = 18) compared to Math1-creERT2::Isl-SmoM2Fli/+ mice (n = 25) (p < 0.0001). No Math1-creERT2::Isl-Pik3caFli/+ (n = 17) or Math1-creERT2::lsl-Pik3caFli/+ (n = 5) mice died because of any symptoms.

3.1.3 Mouse model with PTCH1 mutation

Math1-creERT2::Ptch1Fl/Fl mice show after administration of tamoxifen in all Math1-positive cells and their progeny a homozygous deletion of the Ptch1 gene and a constitutive activation of the SHH pathway. Depending on the time of tamoxifen administration these mice can develop medulloblastoma. Administration between embryonic day E14.5 and postnatal day P8 leads to a tumor incidence of 100% (Yang et al. 2008). The median survival of Math1-creERT2::Ptch1Fl/Fl mice is 127 days (Figure 7m). All tumors display classic histology (Supplementary Figure 25).

Heterozygous deletion (Tamoxifen induction at P3) of the Ptch1 gene (Math1-creERT2::Ptch1Fli/) has no effect on brain development, there is no tumor formation and no abnormalities in survival could be observed (Figure 7).

3.1.4 Mouse model with PTCH1 and PIK3CA mutations

In a sequencing study of Robinson and colleagues (Robinson et al. 2012) the identification of the co-occurrence of mutations in PTCH1 and PIK3CA in medulloblastoma was first described.

Analogous to the previous mouse model, mice with an additional constitutive activated form of PIK3CA were generated. Math1-creERT2::Ptch1Fl/Fl lsl-Pik3caFli/+ and Math1-creERT2::Ptch1Fl/Fl lsl-Pik3caFli/Fl mice showed a faster tumor development compared to Math1-creERT2::Ptch1Fl/Fl mice. Math1-creERT2::Ptch1Fl/Fl lsl-Pik3caFli/+ and Math1-creERT2::Ptch1Fl/Fl lsl-Pik3caFli/Fl mice showed no differences in survival. Kaplan-Meier analysis show that mice with both (Ptch1Fl/Fl and PIK3CA) mutations have a worse survival rate than mice with only a Ptch1Fl/Fl mutation (Figure 7). Heterozygous or homozygous
mutation of PIK3CA alone under the Math1 promoter has no effect on survival. All tumors display classic histology (Supplementary Figure 25).

Heterozygous deletion of the Ptch1 gene and a simultaneous constitutive activated form of PIK3CA (homo- or herterozygous) (Math1-creERT2::Ptch1Fl/ Isl-Pik3caFl/+ and Math1 creERT2::Ptch1Fl/+ Isl-Pik3caFl/Fl) has no effect on survival rate and did not show any symptoms of tumor development.

Figure 7 (a-l) shows an overview of Math1-creERT2::Ptch1Fl/+, Math1-creERT2::Ptch1Fl/Fl, Math1-creERT2::Ptch1Fl/Fl Isl-Pik3caFl/+ and Math1-creERT2::Ptch1Fl/Fl Isl-Pik3caFl/Fl mice: sagittal section H&E and proliferation (Ki67).
Results

Figure 7 Overview Math1-creER<sup>T2</sup>::Ptch1<sup>F/+</sup>, Math1-creER<sup>T2</sup>::Ptch1<sup>F/Fl</sup>, Math1-creER<sup>T2</sup>::Ptch1<sup>F/Fl</sup> Isl-Pik3ca<sup>F/+</sup>, Math1-creER<sup>T2</sup>::Ptch1<sup>F/Fl</sup> Isl-Pik3ca<sup>F/Fl</sup>, Math1-creER<sup>T2</sup>::Isl-Pik3ca<sup>F/+</sup> and Math1-creER<sup>T2</sup>::Isl-
Results

**Pik3ca^{Fl/Fl} mice.** Mice were induced with Tamoxifen at P3 and sacrificed at P30. Left column (a, d, g, j) shows sagittal H&E stains of the whole brain (*scale bar* 1 mm), middle column (b, e, h, k) shows proliferation (Ki67 stains) of the cerebellum (*scale bar* 500 µm) and right column shows higher magnification of Ki67 stains (c, f, i, l) (*scale bar* 10 µm). The Kaplan-Meier analysis (m) shows a sig. lower survival rate for *Math1-creER^{T2::Ptch1^{Fli/Fl} lsl-Pik3ca^{Fl/+}}* (p = 0.0040; n = 5), *Math1-creER^{T2::Ptch1^{Fli/Fl} lsl-Pik3ca^{Fl/Fl}}* (p = 0.0005; n = 5) compared to *Math1-creER^{T2::Ptch1^{Fli/Fl}}* mice (*p* < 0.0001; n = 6). No *Math1-creER^{T2:: lsl-Pik3ca^{Fl/+}}* (n = 17) or *Math1-creER^{T2:: lsl-Pik3ca^{Fl/Fl}}* (n = 5) mice died because of any symptoms.

### 3.1.5 Mouse model with MYCN amplification

A number of observations suggest a role for the proto-oncogene *MYCN* in the pathogenesis of MB. Amplification of *MYCN*, a relatively rare event in MB, correlates with poor outcome (Pfister et al. 2009). Shh promotes the expression and post-transcriptional stabilization of N-Myc in mice (Kenney et al. 2003, Thomas et al. 2009). Brain-specific deletion of murine *N-myc* results in cerebellar dysplasia, suggesting that *N-myc* is critical to proliferation in the developing cerebellum, and that other myc family members cannot compensate for loss of *N-myc* (Knoepfler et al. 2002). Experiments in genetically engineered mice demonstrate that *N-myc* is required for MB development (Hatton et al. 2006), suggesting that human tumors could also arise, in part, through misexpression of *MYCN* (Kenney et al. 2003, Oliver et al. 2003, Hatton et al. 2006, Kessler et al. 2009, Thomas et al. 2009). To date, however, N-myc overexpression (alone or in combination with Gli1, IGF-II, or Bcl-2) has failed to initiate MB in any experimental model (Browd et al. 2006, McCall et al. 2007).

In this work *Math1-creER^{T2::Isl-Nmyc^{Fl/+}}* and *Math1-creER^{T2::Isl-Nmyc^{Fl/Fl}}* were generated. These mouse strains enable an overexpression of Nmyc in cerebellar granule precursors after administration of tamoxifen (at P5). This overexpression did not lead to any tumor formation in our mice (Figure 8) and no symptoms or other abnormalities in the brain could be observed.
3.1.6 Mouse model with MYCN amplification and TP53 mutation

The TP53 gene provides instructions for making a protein called tumor protein p53 (or p53). This protein acts as a tumor suppressor, which means that it regulates cell division by keeping cells from growing and dividing too fast or in an uncontrolled way.

Combined MYC family amplifications and P53 pathway defects (TP53 mutation) commonly emerged at relapse, and all patients in this group died of rapidly progressive disease postrelapse (Hill et al. 2015). These clinical observations and previous modelling of medulloblastoma in mice suggested that aberrant activation of the MYC gene family synergizes with inactivation of p53 or Rb in the genesis of biologically aggressive medulloblastoma (Shakhova et al. 2006, Kawauchi et al. 2012, Pei et al. 2012).

For this project mouse strains with an overexpression on Nmyc and a simultaneous p53 loss of function in cerebellar granule precursors after administration of tamoxifen (at P5) were generated (Math1-creERT2::lsl-NmycFl/+p53Fl/+, Math1-creERT2::lsl-NmycFl/+p53Fl/Fl, Math1-creERT2::lsl-NmycFl/Flp53Fl/+ and Math1-creERT2::lsl-NmycFl/Flp53Fl/Fl mice). Mice with only a p53 loss of function (hetero- and homozygous) were completely inconspicuous.

Recently, the group of Wechsler-Reya developed a similar mouse model with MYCN amplifications. These mice did not develop medulloblastoma, but they developed choroid plexus tumors (personal communication). These tumors arise from brain tissue called the “choroid plexus.” They commonly invade nearby tissue and spread widely via the cerebrospinal fluid. Due to this information we also looked at the choroid plexus.

All these mouse models did neither develop a tumor in the cerebellum, nor abnormalities could be found in the choroid plexus of these animals (Figure 8).

Figure 8 shows an overview of the different genotypes of sagittal sections H&E and proliferation (Ki67).
Figure 8 Overview Math1-creER\textsubscript{T2}::lsl-Nmyc\textsuperscript{Fl/Fl}, Math1-creER\textsubscript{T2}::lsl-Nmyc\textsuperscript{Fl/+}, Math1-creER\textsubscript{T2}::p53\textsuperscript{Fl/Fl}, Math1-creER\textsubscript{T2}::p53\textsuperscript{Fl/+}, Math1-creER\textsubscript{T2}::lsl-Nmyc\textsuperscript{Fl/Fl}p53\textsuperscript{Fl/+}, Math1-creER\textsubscript{T2}::lsl-Nmyc\textsuperscript{Fl/+}p53\textsuperscript{Fl/Fl}, Math1-creER\textsubscript{T2}::lsl-Nmyc\textsuperscript{Fl/Fl}p53\textsuperscript{Fl/+}, Math1-creER\textsubscript{T2}::lsl-Nmyc\textsuperscript{Fl/+}p53\textsuperscript{Fl/Fl}.
Nmyc<sup>FliF</sup>p53<sup>Fli</sup>/ mice and Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/p53<sup>Fli</sup></sup>Fli mice. All mice were induced with Tamoxifen at P5 and sacrificed at P70. Left column (a, d, g, j, m, p, s, v) shows sagittal H&E stains of the whole brain (scale bar 1 mm), right column shows proliferation (Ki67 stains) of the cerebellum (b, e, h, k, n, q, t, w) (scale bar 500 µm) and choroid plexus (c, f, l, o, r, u, x) (scale bar 200 µm).

Survival analysis for Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/</sup> (n = 22), Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/</sup> (n = 11), Math1-creER<sup>T2::p53<sup>FliF</sup>/</sup> (n = 9), Math1-creER<sup>T2::p53<sup>FliF</sup>/</sup> (n = 8), Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/p53<sup>FliF</sup>/</sup> (n = 12), Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/p53<sup>FliF</sup>/</sup> (n = 2), Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/p53<sup>FliF</sup>/</sup> (n = 11) and Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/p53<sup>FliF</sup>/</sup> (n = 4) mice shows no significant difference in prognosis over the different genotypes (Figure 9).

Figure 9 Kaplan-Meier analysis for Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/</sup> (n = 22), Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/</sup> (n = 11), Math1-creER<sup>T2::p53<sup>FliF</sup>/</sup> (n = 9), Math1-creER<sup>T2::p53<sup>FliF</sup>/</sup> (n = 8), Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/p53<sup>FliF</sup>/</sup> (n = 12), Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/p53<sup>FliF</sup>/</sup> (n = 2), Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/p53<sup>FliF</sup>/</sup> (n = 11) and Math1-creER<sup>T2::Isl-Nmyc<sup>FliF</sup>/p53<sup>FliF</sup>/</sup> (n = 4) mice.
3.2 Localization of SHH medulloblastoma in mice depends on the age at its initiation

Approximately, 30% of MBs belong to the SHH subgroup. This subgroup shows a two-peak age distribution, making up the majority of infant (≤ 3 years) and adult (≥ 16 years) MBs, but only a small fraction of childhood (4-15 years) tumors (Kool et al. 2012). Global methylation and gene expression profiles of infant and adult SHH MBs are clearly distinct, although, overall, they belong to the same molecular subgroup (Northcott et al. 2011, Kool et al. 2014). In addition, localization of human SHH MBs differs depending on the age of the patient. For so far unknown reasons, adult SHH MBs are almost exclusively located in cerebellar hemispheres, whereas infant SHH MBs often grow in the vermis (Wefers et al. 2014). Since the genetic makeup is known to be different in infant and adult MB (Kool et al. 2014), it appears possible that the anatomical localization is dependent on the tumor’s driver mutations. On the other hand, it appears possible that the susceptibility of different cell types for oncogenic transformation is dependent on a distinct developmental time frame, but independent of the tumor-initiating driver mutation. To test this hypothesis in a systematic way, we used Math1-GFP, Ptc+/− mice, which develop green fluorescent SHH MB (Goodrich et al. 1997, Oliver et al. 2005). Importantly, in contrast to SUFU or SMO mutations that predominantly occur in SHH MB of infants and adults, respectively, PTCH1 mutations are equally detected in both age groups of human SHH MB (Kool et al. 2014, Pöschl et al. 2014). So, PTCH1 mutations per se do not appear to predispose for a specific tumor localization.

First, we analysed the localization of Math1-GFP, Ptc+/− MBs in the cerebellum at different time points. The localization of a tumor (as defined as >2 mm in size) was categorized according to the involvement of vermis and hemispheres, the involvement of rostral and caudal cerebellar regions, and its growth into the fourth ventricle (Figure 10).
We confirmed expression of Sox2 in all tumors that were included in our statistics, as preneoplastic lesions and real tumors have quite different gene expression profiles, and Sox2 is at the top of the list of the genes that are expressed in tumors, but not in normal granule cell precursors or preneoplastic lesions (Oliver et al. 2005) (Figure 11).

We analysed 81 5-week-old and 34 10-week-old Math1-GFP,Ptc<sup>+</sup>−/− mice (Figure 13a). In the 5-week-old group, 44/81 animals (54%) had no MB, 23/81 (29%) developed one tumor, 13/81 (16%) developed two separable tumors, and 1/81 (1%) developed three separable tumors within the cerebellum. Only 7/34 animals (20%) in the 10-week-old group did not develop any MB, 4/34 (12%) had 2 separable tumors, and 23/34 (68%) developed one tumor (p < 0.001). Hence, the overall tumor incidence was 46% in 5-week-old mice and 80% in 10-week-old mice. This implies that a good number of tumors
develop after 5 weeks of age. Representative examples of MB in 5- or 10-week-old *Math1-GFP,Ptc*<sup>+/−</sup> mice are given in Figure 12.

![Representative fluorescent images and H&E stains of MB in *Math1-GFP,Ptc*<sup>+/−</sup> mice at different time points. Arrows point to tumors, which display classic histology at both ages. High magnifications are shown on the right. Scale bars correspond to 2 mm for low-power images and 10 µm for high-power images.](image)

Every tumor was then categorized according to its main localization in the cerebellum (Figure 13 b). 31/52 MBs of the 5-week-old *Math1-GFP,Ptc*<sup>+/−</sup> mice (60 %) exclusively grew in the hemispheres, but 9/52 tumors (17 %) were restricted to the vermis. In contrast, all 31 tumors in the 10-week-old group grew in the hemispheres with 15/31 (48 %) being exclusively in the hemispheres (p < 0.01). This implies that tumors in the vermis do not develop at later stages and must develop in embryonic or early postnatal stages. Otherwise, one would clearly expect MBs that exclusively occupy the vermis at 10 weeks of age (Ohli et al. 2015).

Furthermore, we analysed the main vertical MB localization (Figure 13 c).
Almost half of the tumors of the 5-week-old mice (25/52, 48%) grew only caudal, whereas only 11/52 (21%) grew exclusively rostral. In the 10-week-old group, 8/31 (26%) grew only caudal, 2/31 (6%) grew only rostral, but the majority of these tumors extended rostro-caudal (21/31, 68%) (p < 0.01). Moreover, 6/52 (12%) of the 5-week-old MBs and 10/31 (32%) of the 10-week-old MBs expanded into the fourth ventricle (Figure 13 d, p = 0.02).

To confirm the time-dependent localization of SHH medulloblastoma in an independent model, we used Math1-creERT2::Isl-SmoM2Fl/+ mice that were Tamoxifen-induced at E14.5 or P5. To make sure that the targeted cells under both conditions are comparable, we first performed lineage tracing analyses using Math1-creERT2::tdTomato mice and found an equal distribution of creERT2-mediated recombination, as indicated by RFP staining throughout the vermis and the hemispheres for both time points of Tamoxifen induction (Figure 14 a). Therefore mice from the Math1-creERT2 strain were paired with mice which carry a sequence for a red fluorescent protein (tdTomato, variant of RFP). With the Cre-LoxP-system it is possible through, the cre-mediated recombination, to cut out specific
sequences lying between two loxP-sequences. In the system used here, the sequence which is cut out is a polyadenylation sequence, thus a stop-codon. After the loss of the stop-codon, the expression of the coding reporter genes could take place. This induced modification in the protein expression also manifest after the cell division and independent of further differentiation of progeny. Thus, every daughter cell of a primary recombinated cell further on express RFP. Due to the traceability of the fate of a single cell and populations with this system, it is called fate-mapping or lineage tracing analysis.

Tumor growth in \textit{Math1}\textsuperscript{-}\textit{creERT2::Isl-SmoM2}\textsuperscript{fl/+} mice was then analysed in the vermis and hemispheres at P14 or P26, i.e., 3 weeks after Tamoxifen induction (Figure 14 b). In agreement with the data above, we did not find anatomical differences in the tumor growth when induced at E14.5. However, animals induced postnatally (P5) developed tumors, the growth of which was clearly pronounced in the hemispheres (Figure 14 c). There was significantly less tumor burden in the vermis of P5-induced animals compared to embryonically induced mice. Together, these results fit well to the distribution of SHH MBs in humans (Wefers et al. 2014) and indicate that the localization of SHH MBs is dependent on the time-restricted susceptibility of granule cell precursors in defined cerebellar compartments, and independent of the type of tumor-initiating driver mutation.
Results

Figure 14 (a) RFP stainings indicating equally distributed recombination in granule cells of Math1-creER\textsuperscript{T2::tdTomato} mice. (b) H&E stainings of embryonically and postnatally induced Math1-creER\textsuperscript{T2::Isl-SmoM2\textsuperscript{Ft/+}} mice. Arrows indicate tumor areas in the vermis and hemisphere. (c) Quantification of the relative tumor area of embryonically and postnatally induced Math1-creER\textsuperscript{T2::Isl-SmoM2\textsuperscript{Ft/+}} mice in different cerebellar compartments. Scale bars correspond to 2 mm for low-power image (right image in a and both images in b), 500 µm (left image in a). Bars show mean with SEM. ns not significant, ** p < 0.01, *** p < 0.001.
3.3 Effects of Wnt/β-catenin signalling on Shh-dependent medulloblastoma formation

Shh is crucial for the proliferation of cerebellar granule neuron precursors (GNPs) (Wechsler-Reya and Scott 1999), but constitutive activation of the Shh pathway in GNPs leads to medulloblastoma (Schüller et al. 2008). In contrast, canonical Wnt/β-catenin signalling is required for the maturation of GNPs, and constitutive activation of this pathway causes significant proliferation deficits (Lorenz et al. 2011). To develop new therapeutic strategies for patients with Shh-medulloblastoma, we asked whether the physiological roles of Wnt/β-catenin signalling can be utilized to treat Shh-medulloblastomas that have developed from GNPs.

To analyse the effect of Wnt/β-catenin-activation, primary cell culture experiments were performed. At the time point of the biggest clonal expansion of the granule cell precursors (p5-p7) the Shh-activating SmoM2-mutation and the Wnt-activating form of β-catenin were introduced by viral inserted cre-recombinase. SmoM2\(^{Fl/+}\), Ctnnb1\((ex3)^{Fl/+}\), or SmoM2\(^{Fl/+}\)Ctnnb1\((ex3)^{Fl/+}\) cerebellar GNPs were cultured as described (Lorenz et al. 2011) and transduced with Cre-IRES-GFP viruses. Cre treatment of SmoM2\(^{Fl/+}\) cerebellar GNPs led to a 2.6-fold proliferation increase due to active Shh signalling (Figure 15, \(p = 0.008\)). Here, proliferation is the fraction of BrdU\(^+\) cells among GFP\(^+\) granule cells [%].
Results

Figure 15 Cultured cerebellar GNPs from mice of indicated genotypes were transduced with control IRES-GFP virus or Cre-IRES-GFP virus to recombine alleles. Wnt/ß-catenin-activation significantly reduced proliferation of both normal GNPs and SmoM2Fl/+ -transformed GNPs. Bars show mean with SEM. ns not significant. * p < 0.05, ** p < 0.01.

Wnt pathway activation in Ctnnb1(ex3)Fl/+ GNPs reduced proliferation to 52% (Figure 15, p = 0.029). Importantly, Wnt-activation significantly decreased oncogenic GNP proliferation driven by SmoM2 (SmoM2Fl/+ Ctnnb1(ex3)Fl/+ condition, p = 0.028), and cells reached proliferation levels similar to the wild type. Thus, the proliferation of Shh-medulloblastoma cells may be blocked through canonical Wnt signalling. These results extend previous studies showing inhibitory effects of Wnt3a through non-canonical Wnt signalling on GNPs (Anne et al. 2013).

Next, we introduced a Ctnnb1(ex3)Fl/+ allele into Math1-cre::SmoM2Fl/+ mice, a model for Shh-medulloblastoma (Schüller et al. 2008). Activation of the Shh pathway in Math1-positive GNPs resulted in a thickened external granule cell layer (EGL) and medulloblastoma (Figure 16 a, b; Supplementary Figure 26). Additional activation of the Wnt-pathway (Math1-cre::SmoM2Fl/+ Ctnnb1(ex3)Fl/+ mice) notably reduced growth of medulloblastoma and resulted in decreased cerebellar size (Figure 16 a, c; Supplementary Figure 26). Importantly, Math1-cre::SmoM2Fl/+ Ctnnb1(ex3)Fl/+ mice (n = 16) survived significantly longer than Math1-cre::SmoM2Fl/+ animals (n = 53, p < 0.0001, (Figure 16 d)).
Results

Figure 16 (a-c) Sagittal cerebellar sections. Shh pathway activation in Math1-positive cerebellar GNPs resulted in the formation of medulloblastoma (b). Co-activation of the Wnt/β-catenin signalling pathway [Math1-cre::SmoM2Fl/+Ctnnb1(ex3)Fl/+ mice] reduced growth of medulloblastoma and led to a decreased cerebellar size (c). (d) Math1-cre::SmoM2Fl/+Ctnnb1(ex3)Fl/+ mice displayed a significantly prolonged survival. ML molecular layer, PL Purkinje cell layer, IGL internal granule cell layer

In order to just analyse cells, that were transduced with Cre-IRES-GFP virus, we used FACS (fluorescence-activated cell sorting) to isolate GFP+ cells from SmoM2Fl/+ or SmoM2Fl/+Ctnnb1(ex3)Fl/+ GNP cultures after Cre-IRES-GFP virus treatment. Looking at different targets of the Shh and the Wnt pathway, quantitative RT-PCR analysis was performed. It could be confirmed that the mRNA level of Axin2, a direct target of the Wnt pathway, was upregulated in SmoM2Fl/+Ctnnb1(ex3)Fl/+ GNPs, whereas the mRNA level of Gli1, a downstream target of the Shh pathway, was downregulated (Figure 17).
Hence, active canonical Wnt signalling is a potent inhibitor of the Shh pathway in medulloblastoma. Pharmacologically, activation of Wnt/β-catenin signalling can be achieved via inhibition of GSK3β, an upstream player of the pathway (Rubinfeld et al. 1996). Primary cell culture was used for cre treatment of cerebellar GSK3β Fl/Fl GNPs (Jaworski et al. 2011). A significant decrease in proliferation was achieved when GSK3β was knocked out in vitro (Figure 18, p = 0.041).

Figure 18 GSK3β knockout resulted in significantly reduced proliferation of GNPs. (a) Cultured cerebellar granule neuron precursors (GNPs) from GSK3β mice (P5-P7) were transduced with IRES-GFP (control) and Cre-IRES-GFP virus to recombine alleles. GFP staining (green) shows recombined cells, BrdU staining (red) shows proliferating cells. Knockout of GSK3β led to decreased proliferation of transduced cerebellar GNPs (arrows point to proliferating cells that have undergone successful recombination). (b) Quantifications for...
Results

61

results shown in (a) including control experiments with IRES-GFP virus. Bars show mean of three individual experiments. Knockout of GSK3β significantly reduced proliferation of cerebellar GNPs (p = 0.041). Bars show mean with SEM. ns not significant, * p < 0.05 (t-test).

3.3.1 In vitro treatment with the Wnt agonist lithium chloride

Lithium is a chemical element with psychotropic properties, which is commonly used to treat bipolar disorder as well as other psychiatric diseases (Geddes et al. 2004, Storosum et al. 2007), although the underpinnings of its biochemical effects remain only partially understood (Alda 2015). Among others, Lithium has been shown to act as an inhibitor of Glycogen synthase kinase 3 beta, a serine/threonine kinase also known as GSK3B (Klein and Melton 1996, Stambolic et al. 1996, O’Brien and Klein 2009). GSK3B is part of the so-called cytoplasmic destruction complex together with Axin, Adenomatosis polyposis coli (APC), Dishevelled (Dvl), the CK1-Kinase group and GSK3A (Clevers 2006). This protein complex leads to phosphorylation, ubiquitination, and therefore proteasomal degradation of β-Catenin in the cytoplasm (Aberle et al. 1997, Willert et al. 1999). Hence, Lithium is able to activate the Wnt/β-Catenin pathway by inhibiting GSK3B and therefore preventing phosphorylation and subsequent degradation of β-Catenin (Stambolic et al. 1996). Previous results demonstrated an inhibitory effect of constitutional Wnt/β-Catenin activation on Shh-dependent medulloblastoma formation in Math1-cre::SmoM2fl/+;Ctnnb1(ex3)fl/+ mice (Pöschl et al. 2014). In order to examine, whether a Wnt agonist might represent a suitable treatment option for patients with Shh-dependent medulloblastoma, a series of in vitro experiments was conducted, in order to inquire a possible inhibitory effect of lithium chloride (LiCl), a highly soluble lithium salt, which has been suggested to act as a Wnt/β-Catenin activator in the cerebellum (Lancaster et al. 2011). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were carried out to investigate, whether the treatment of medulloblastoma tumor cells with LiCl would result in a decrease of cell viability in vitro (Figure 19).
Figure 19 Lithium chloride (LiCl) reduces medulloblastoma cell viability in vitro. Colorimetric MTT assays after 24h in vitro treatment with 10 mM LiCl decreased cell viability of granule neuron precursor cells and medulloblastoma tumor cells from Math1-cre::SmoM2Fl/+ and Ptch1+/− mice, when compared to HEK293T, UW473 and GL261 control cells. p < 0.01, t-test. Bars show mean with SEM. ns not significant. **p < 0.01.

Granule neuron precursor cells (GNPs), from which Shh-dependent medulloblastoma arise (Schüller et al. 2008), as well as medulloblastoma tumor cells from Math1-cre::SmoM2Fl/+ and Ptch1+/− mice, two well-known murine models for Shh-induced medulloblastoma (Goodrich et al. 1997, Schüller et al. 2008), were isolated and put into primary culture for 16 hours. These cells were then treated with 10 mM LiCl for 24 hours in parallel with the fibroblast line HEK293T, the (non-SHH) medulloblastoma cell line UW473 and the glioma cell line GL261 cell lines, which served as Shh-independent control cells. As a result, LiCl lead to a significant decrease of cell viability in Shh-dependent GNPs and tumor cells from Math1-cre::SmoM2Fl/+ and Ptch1+/− mice when compared to control cells (p < 0.01) (Figure 19). Thus, LiCl is able to inhibit proliferation of Shh-associated medulloblastoma cells in vitro.
3.4 Preclinical treatment of mouse models of SHH medulloblastoma with Hh inhibitors

![Diagram of preclinical treatment process](image)

Figure 20 Overview over treatment of Shh-associated mouse models. Using CreERT2 systems, recombination will be induced by a single i.p. shot of 1 mg Tamoxifen at postnatal day 3 or 5. The inducible conditional transgenic mice will receive primary treatment starting at day 30 using LDE225 (30 mg/kg/day i.p., 5 days per week, treatment duration 21 days). Once mice become symptomatic they will be sacrificed. One half of the brain will be processed for histology and tumor tissue will be harvested from the other second half for molecular analyses. Tumor samples with and without LDE225 treatment will be analysed and compared.

Targeted therapies as a novel treatment modality for MB patients are especially intriguing for primary and relapsed SHH-MB. As more drugs targeting the Hh pathway become available and enter clinical trials, it is important to know how to stratify the patients for different drugs in order to maximize response rates and to prevent unnecessary treatment failures.

Mouse models for medulloblastoma can be treated with SMO inhibitors that have already entered clinical trials and drugs targeting at the level of GLI, such as arsenic trioxide (ATO), bromodomain inhibitors like JQ1 or other more recently developed specific GLI inhibitors, such as GANT61.

Transgenic mice are induced with tamoxifen and then randomly divided in two groups (vehicle/with treatment). After tumor development, mice were sacrificed und tumor tissue was harvested for molecular analyses (Figure 20).
3.4.1 LDE225 treatment

Here we focus on the SMO antagonist LDE225. LDE225 (also known as Sonidegib; Novartis Pharma) is a synthetic, highly potent and selective, small molecule clinical SMO inhibitor resulting from the optimisation as a hit arising from a high throughput screening phenotypic assay designed to identify SMO inhibitors. LDE225 interacts directly with SMO, in a similar fashion to cyclopamine, to reduce expression of downstream Hh signalling targets (Pan et al. 2010). LDE225 is effective in various Hh-dependent tumor models and inhibits downstream expression of Hh targets in cell lines, in vivo animal models and in patients, and is currently under clinical trial investigation both as a single agent (Rodon et al. 2014) and in combination (reviewed in (Brechbiel et al. 2014)).

All mice are monitored for treatment response, development of resistance, and overall survival.

3.4.1.1 LDE225 treatment in Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} mice

To determine the effects of LDE225 treatment on tumor growth in vivo, Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} mice at P30 were randomized to receive either vehicle or LDE225 (30 mg/kg/day) five times per week for three weeks and assessed for tumor development. Tumor size was substantially reduced in LDE225-treated Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} mice compared to the control condition. LDE225 treatment resulted in a significantly extended lifespan of Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} mice (Figure 21 a). After three weeks of LDE225 treatment there is a significant reduction in proliferation or BrdU\textsuperscript{+} cells in LDE225-treated tumors of Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} mice (Figure 21 b). There is no change in morphology after treatment compared to untreated Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} tumors (data not shown).
Figure 21 (a) Kaplan-Meier survival curve illustrating overall survival of Math1-creER\textsuperscript{T2}:Isl-SmoM2\textsuperscript{F/+} mice in LDE225 treatment cohort. All mice were induced to tumor formation at P3 by tamoxifen and treated with vehicle or LDE225 from P30 to P51. LDE225 treatment leads to a significant improvement of survival rate (log rank test, p < 0.0001); (b) Quantification of the fraction of BrdU\textsuperscript{+} tumor cells at P51 in Math1-creER\textsuperscript{T2}:Isl-SmoM2\textsuperscript{F/+} mice treated with vehicle or LDE225 for 21 days (n = 3, mean ± SEM, t-test), scale bar 20 \(\mu\text{m}\). *** p < 0.001.
3.4.1.2 LDE225 treatment in Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} Isl-Pik3ca\textsuperscript{Fl/+} mice

Figure 22 Comparison of Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} and Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} Isl-Pik3ca\textsuperscript{Fl/+} mice. H&E (a, c) scale bar 1 mm; Ki67 (b, d) scale bar 500 µm.

To determine the effects of LDE225 treatment on tumor growth in a similar mouse model (described above) with an additional mutation in Pik3ca, Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} Isl-Pik3ca\textsuperscript{Fl/+} mice were treated in a similar way like Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} mice. As Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} Isl-Pik3ca\textsuperscript{Fl/+} mice had a lower median survival and much faster tumor growth than Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} mice, Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} Isl-Pik3ca\textsuperscript{Fl/+} mice often died during the 3 week long treatment phase. Therefore treatment start was moved up from P30 to P20 (Figure 22). As first symptoms normally occurred around P20 in Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} Isl-Pik3ca\textsuperscript{Fl/+} mice and in Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} mice around P30, this shifted time point of treatment start makes the treatment of this two mouse strains more comparable (Figure 22). Both treatment groups had no significant improvement of survival compared to the control group. However, Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} Isl-Pik3ca\textsuperscript{Fl/+} mice with an earlier treatment start (P20) have a better prognosis than Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} Isl-Pik3ca\textsuperscript{Fl/+} mice with a
later treatment start (P30) (p = 0.0065) (Figure 23 a). However, after three weeks of LDE225 treatment there is a significant reduction in BrdU+ cells in LDE225-treated tumors of Math1-creERT2::Isl-SmoM2^{fl/+} Isl-Pik3ca^{fl/+} mice with treatment start at P20 as well as with treatment start at P30 compared to the control group (Figure 23 b). LDE225 treatment does not change the morphology of Math1-creERT2::Isl-SmoM2^{fl/+} Isl-Pik3ca^{fl/+} tumors compared to untreated tumors (data not shown).

Figure 23 (a) Kaplan-Meier survival curve illustrating overall survival of Math1-creERT2::Isl-SmoM2^{fl/+} Isl-Pik3ca^{fl/+} mice in LDE225 treatment cohort. All mice were induced to tumor formation at P3 by tamoxifen and treated with vehicle or LDE225 from P30 to P51 or P20 to P41. Math1-creERT2::Isl-SmoM2^{fl/+} Isl-Pik3ca^{fl/+} LDE225 (treatment start P20) (n = 10) vs Math1-creERT2::Isl-SmoM2^{fl/+} Isl-Pik3ca^{fl/+} vehicle (n = 13) (p = 0.0651); Math1-creERT2::Isl-SmoM2^{fl/+} Isl-Pik3ca^{fl/+} LDE225 (treatment start P30) (n = 9) vs Math1-creERT2::Isl-SmoM2^{fl/+} Isl-Pik3ca^{fl/+} vehicle (n = 13) (p = 0.2982); Math1-creERT2::Isl-SmoM2^{fl/+} Isl-Pik3ca^{fl/+}
LDE225 (treatment start P20) (n = 10) vs Math1-creERT2::Isl-SmoM2Fl/+ Isl-Pik3caFl/+ LDE225 (treatment start P30) (n = 9) (p = 0.0065); (b) Quantification of the fraction of BrdU⁺ tumor cells at P51 (for vehicle and LDE225 (P30)) and at P41 (for LDE225 (P20)) in Math1-creERT2::Isl-SmoM2Fl/+ Isl-Pik3caFl/+ mice treated with vehicle or LDE225 for 21 days (n = 3, mean ± SEM, t-test), scale bar 20 μm. * p < 0.05, ** p < 0.01, *** p < 0.001.

3.4.1.3 LDE225 treatment in Math1-creERT2::Ptch1Fl/FI mice

This mouse strain was also treated with the same dosage (30 mg/kg/day) and duration (5 times per week, for 3 weeks) of LDE225. Here treatment start was P30. LDE225 treatment leads to a significant better survival rate (p = 0.0198) compared to the control group (Figure 24 a). After three weeks of LDE225 treatment there is a significant reduction in BrdU⁺ cells in LDE225-treated tumors of Math1-creERT2::Ptch1Fl/FI mice (Figure 24 b). The treatment has no effect on the morphology of Math1-creERT2::Ptch1Fl/FI tumors compared to untreated tumors (data not shown).
Figure 24 (a) Kaplan-Meier survival curve illustrating overall survival of \textit{Math1-creER}\textsuperscript{T2::Ptch1\textsuperscript{F/F}} mice in LDE225 treatment cohort. All mice were induced to tumor formation at P3 by tamoxifen and treated with vehicle or LDE225 from P30 to P51. \textit{Math1-creER}\textsuperscript{T2::Ptch1\textsuperscript{F/F}} vehicle (n = 14) vs \textit{Math1-creER}\textsuperscript{T2::Ptch1\textsuperscript{F/F}} LDE225 (n = 7) (p = 0.0198); (b) Quantification of the fraction of BrdU\textsuperscript{+} tumor cells at P51 in \textit{Math1-creER}\textsuperscript{T2::Ptch1\textsuperscript{F/F}} mice treated with vehicle or LDE225 for 21 days (n = 3, mean ± SEM, \textit{t}-test), scale bar 20 \textmu m. *** p < 0.001.
4 Discussion

MB is a collection of clinically and molecularly distinct tumor subgroups that arise either in the cerebellum or brainstem (Louis et al. 2007, Schüller et al. 2008, Grammel et al. 2012, Taylor et al. 2012). In children they comprise the most frequent embryonal brain tumor, whereas in adults the disease is relatively rare, accounting for about 1% of all intracranial malignancies (Louis et al. 2007). Current therapy regimens including surgery, craniospinal radiotherapy, and chemotherapy, may cure 70-80% of patients with MB. Most survivors, however, suffer from long-term sequelae as a result of the intensive treatment, demonstrating that less toxic treatments are urgently needed. Molecular analyses have shown that there are four major MB subgroups (WNT, Sonic Hedgehog (SHH), Group 3, Group 4) (Taylor et al. 2012). But this is just a rough classification as a considerable amount of heterogeneity was already described within a single molecular subgroup. Specifically for human SHH-associated MB, differences on the molecular level and also in clinical parameters were described for infants and adult patients (Northcott et al. 2011). Curiously, Shh-MB occur in a bimodal distribution, making up the majority of infant (≤ 3 years) and adult (≥ 16 years) MBs, but only a very small fraction of childhood (4-15 years) tumors. This unusual bimodal age distribution also suggests the existence of Shh-subgroup heterogeneity.

As targeted therapies for MB patients are novel strategies for treatment, this project focused on different mouse models for analysing Shh-associated MBs in more detail.

4.1 Localization of SHH medulloblastoma in mice

In order to unravel a possible relation between MB biology and localization, and to provide novel hints regarding the cellular origin of MB, Math1-GFP,Ptc"−/− mice, which develop green fluorescent SHH MBs, were analysed regarding tumor localization. MB occurring in Ptc"−/− mice or Smo-based mouse models resemble the human counterparty in an excellent manner (Pöschl et al. 2014). For so far unknown reasons, human adult SHH MBs are almost exclusively located in cerebellar hemispheres, whereas human infant SHH
MBs often grew in the vermis (Wefers et al. 2014). Here we show in mice that 60% of the younger group (5-week-old) had tumors that exclusively grew in the hemispheres, but 17% were restricted to the vermis. In contrast, in the older group (10-week-old) all tumors grew in the hemispheres and no single tumor was restricted to the vermis (Figure 13). This leads to the suggestion that tumors in the vermis do not develop at later stages and must develop in embryonic or early postnatal stages. Otherwise, one would clearly expect MBs that exclusively occupy the vermis also in the older group. To confirm the time-dependent localization of SHH MB, another Shh-associated mouse model (\textit{Math1-creER}^{T2::Isl-SmoM2^{FL/+}}) was used (Figure 14). It could be demonstrated that there is less tumor burden in the vermis of postnatal-induced mice compared to embryonically induced mice. Using well-established mouse models we showed here that oncogenic transformation of granule cell precursors at early developmental time points may result in the formation of midline and hemispheric medulloblastoma. On the other side, oncogenic transformation at later developmental time points exclusively results in the formation of hemispheric medulloblastoma. These results perfectly match to the recently published distribution of SHH MBs in humans (Wefers et al. 2014) and indicate that the localization of SHH MBs is dependent on the time-restricted susceptibility of granule cell precursors in defined cerebellar compartments, and independent of the type of tumor-initiating driver mutation.

The tumor incidence of the \textit{Math1-GFP,Ptc}^{+/-} mice used here was clearly higher as described before (Figure 13 a). The 5-week old group show a tumor incidence of 46% and 10-week old mice 80%, whereas Oliver and colleagues only described a tumor incidence of 14-20% of \textit{patched} heterozygotes (Oliver et al. 2005). The rather high incidence in this study here could be explained as it is known that the tumor incidence (and spectrum) in \textit{Ptc}^{+/-} mice may vary from colony to colony.

The time point of tumor analysis (as for \textit{Math1-GFP,Ptc}^{+/-} mice) and tumor induction (as for \textit{Math1-creER}^{T2::Isl-SmoM2^{FL/+}} mice) is a crucial point with some limitations. Apart from tumors that eventually develop, normal granule neuron precursors also express Math1 and will hence shine green in \textit{Math1-GFP} mice. Thus, mice with 3 weeks of age or even younger are not suited for our experiments, because granule neuron precursors are still present and one would detect green fluorescence all over the cerebellum. Furthermore, SHH MB occur either in infancy (predominantly in midline structures) or in adulthood.
(predominantly in cerebellar hemispheres). 1 year in humans approximately corresponds to 3 weeks of age in mice (ten Donkelaar et al. 2006, Manto et al. 2013). While 3 weeks is too early for tumor analysis in mice, 5 weeks of age appears appropriate and still corresponds to human infancy. 10 weeks of age in mice were chosen, since this is well after mice become fertile, which corresponds to the age of 16-20 years of humans. This exactly covers the second peak of incidence for human SHH medulloblastoma. Mice older than 10 weeks of age appear inappropriate, since we would lose too many mice, which die between 10 and 20 weeks of age. Furthermore, tumors may have become huge, so that it is very hard, if not impossible to judge, whether a tumor started in the vermis or in one of the hemispheres. For the tamoxifen application in Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} mice, there is a theoretical time frame between E14.5 and P8 with 100% tumor incidence (Yang et al. 2008). While it is very hard to estimate, at which time point tumor initiation occurs in humans, we thought that choosing time points for tamoxifen induction that are far away from each other would be good to detect any possible differences in tumor localization.

4.2 Wnt/\beta-catenin signalling and Shh-dependent medulloblastoma

Survival of children with relapsed or refractory MB remains remarkably poor despite aggressive treatment protocols and cure is a rare exception for these patients (Jones et al. 2012). This highlights the urgent medical need to identify and validate novel molecular targets that can be exploited for the treatment of these patients in order to improve their dismal prognosis. Since conventional therapies and risk stratification have already been optimized in recent years, molecular targeted therapies are currently considered to open promising perspectives for more efficient therapies.

The SHH pathway interacts with different other pathways. The Wnt/beta-catenin and the Hedgehog (Hh) pathway interact in various cell types while eliciting opposing or synergistic cellular effects (see 1.3.3.3). Previous studies showed that Wnt/\beta-Catenin activation is able to inhibit the Shh-driven proliferation of cerebellar granule neuron precursor cells (CGNPs) (Lorenz et al. 2011) as well as tumor growth in Shh-dependent medulloblastoma (Pöschl et al. 2014). Our in vitro analyses in GNPs of SmoM2\textsuperscript{Fl/+}, Ctnnb1(ex3)\textsuperscript{Fl/+} and SmoM2\textsuperscript{Fl/+} Ctnnb1(ex3)\textsuperscript{Fl/+} mice could demonstrate that Shh signalling
leads to an increase of proliferation, Wnt signalling to a decrease of proliferation, and Wnt signalling decreases the proliferation in the increased condition of Shh signalling. Thus, the proliferation of Shh-medulloblastoma cells may be blocked through canonical Wnt signalling. These results extend previous studies showing inhibitory effects of Wnt3a through non-canonical Wnt signalling on GNPs (Anne et al. 2013). Furthermore, we could demonstrate that the Wnt target Axin2 is upregulated and the Shh target Gli1 is downregulated in GFP+ cells of SmoM2Fl/+Ctnnb1(ex3)Fl/+ GNP cultures. In addition, β-catenin was identified as a GSK3 substrate: GSK3-mediated phosphorylation triggers β-catenin destabilization (Peifer et al. 1994, Yost et al. 1996). This finding thus established a central role for GSK3 in Wnt/β-catenin signalling. Studies since then have revealed multifaceted roles of this kinase in Wnt signal transduction. Although the detailed mechanisms for GSK3 regulation during Wnt signal transduction remain incompletely understood, it is clear that Wnt-mediated GSK3 regulation does not utilize the same phosphorylation events as in AKT signalling (Yuan et al. 1999, Ding et al. 2000). Recent advances indicate that GSK3 also plays a positive role in Wnt signal transduction by phosphorylating the Wnt receptors low density lipoprotein receptor-related protein (LRP5/6) and provide new mechanisms for the suppression of GSK3 activity by Wnt in β-catenin stabilization. Furthermore, GSK3 mediates crosstalk between signalling pathways and β-catenin-independent downstream signalling from Wnt. Hence, active canonical Wnt signalling is a potent inhibitor of the Shh pathway in medulloblastoma. Pharmacologically, activation of Wnt/β-catenin signalling can be achieved via inhibition of GSK3β. Cre treatment of cerebellar GSK3βFl/Fl GNP cultures significantly decreased proliferation (Figure 18).

With the intent of investigating, whether the newly discovered inhibitory effect of Wnt over Shh might represent a novel approach in the therapy of Shh-associated medulloblastoma, in vitro experiments on medulloblastoma cells were conducted using Lithium, a well-known Wnt activator (Stambolic et al. 1996, Lancaster et al. 2011). Results showed that Lithium is able to reduce cell viability of CGNPs and medulloblastoma tumor cells from Math1-cre::SmoM2Fl/+ and Pch1+/- mice in vitro (Figure 19). Therefore, considering the encouraging in vitro results, it is possible to propose the hypothesis that Wnt activators may represent a valuable therapeutic option for Shh-associated medulloblastoma. Further investigations adopting different dosages of Lithium and other Wnt-activating substances appear therefore to be required.
4.3 Establishment and characterization of mouse models

Besides the Wnt pathway, there are some other pathways that interact with the SHH pathway and components of these pathways may represent venues for further investigation, especially in the context of combination therapy with the SHH pathway. P53 is one of it. In the light of the observation from Kool et al. 2014 that TP53 was enriched in pediatric (ages 4-17) MBs, the role of p53 in MB should be examined closely. P53 status is important in the prognosis of patients with SHH-medulloblastoma, as well as disease incidence. A cohort study suggested that TP53 status plays a critical role in survival status of patients, with five-year survival rates differing significantly between 41% and 81%, respectively, for SHH-medulloblastoma patients with and without TP53 mutations (Zhukova et al. 2013). In this study we tried to develop a tumor model based on MYCN amplification and TP53 mutation in cerebellar granule precursors, as this combination commonly occurred in the human system at relapse of MB patients (Hill et al. 2015). As no tumor development or any other lesions in the brain could be observed (Figure 8), one explanation could be that the tumor incidence of our mouse model is so low, that we could not detect one tumor, compared to our Math1-creERT2::Isl-SmoM2F/ model, in which penetrance was 100%. This suggests that, in general, Cre-mediated excision should be sufficient to achieve a tumor penetrance of 100%, but also that MYCN and P53 expression may be variable and that a threshold sufficient for tumor development may not be reached in tumor-free Math1-creERT2::Isl-Nmyc p53 (homo- and heterozygous) mice. Alternatively, additional hits that have not occurred in tumor-free mice may be required for tumor development. However, a potential “second hit” could not be identified yet.

MYC family oncogenes are amplified in ~10% of medulloblastoma tumors and amplifications correlates with poor survival (Huse and Holland 2010, Kool et al. 2012, Korshunov et al. 2012). MYCN is highly expressed in two of the medulloblastoma subgroups (SHH group and Group 4 tumors) (Taylor et al. 2012). To date, however, N-myc overexpression (alone or in combination with Gli1, IGF-II, or Bcl-2) has failed to initiate MB in any experimental model (Browd et al. 2006, McCall et al. 2007). Interestingly, a model of MB where murine-derived allografted cells are implanted into
nude mice could be developed (Cage et al. 2015). Since PI3K stabilizes MYCN, PI3K inhibitors were used to drive degradation. It could be shown that these inhibitors can drive apoptosis in MYCN-driven cancers.

PI3K/AKT signalling is also shown to crosstalk with Hh signalling. The activity of Gli1 is crucial determined by its protein stability and localization. Several kinases were known to modulate Gli1 protein stability and localization. Treatment of melanoma cells with AKT1 inhibitors decreased the number of cells with nuclear Gli1 labelling, while increasing the number of cells with cytoplasmic Gli1 labelling, suggesting that AKT1 may be required for Gli1 nuclear localization and transcriptional activity (Stecca et al. 2007). However, as shown in neuroblastoma, Gli1 activity is suppressed by AKT2 which phosphorylates GSK3β, leading to GSK3β stabilizing the inhibitory SUFU/GLI1 complex (Paul et al. 2013). As Gli1 represents downstream SHH signalling, it is an attractive target for specific SHH mediated MB. Inhibition of its activating kinases or activation of its inhibitory kinases can be explored. Here, we combined the already existing and well described SHH mouse models Math1-creERT2::Isl-SmoM2Fl/+(see 3.1.1) and Math1-creERT2::Ptch1Fl/Fl (see 3.1.3) with a PIK3CA mutation (Math1-creERT2::Isl-SmoM2Fl/+ Isl-Pik3caFl/+ , Math1-creERT2::Ptch1Fl/Fl Isl-Pik3caFl/+ and Math1-creERT2::Ptch1Fl/Fl Isl-Pik3caFl/Fl mice). To analyse the effect of an acute PIK3CA mutation in the view of oncogenic transformation, these inducible tumor models were generated, which enable a conditional activation of the Shh pathway in postnatal granule cell precursors and a simultaneous constitutive activated form of PIK3CA. The additional PIK3CA mutation shows an increase in proliferation, leads to a faster tumor development and therefore a significantly worse survival prognosis (Figure 6, Figure 7). Hh and PI3K signalling appear to synergize in promoting proliferation of cerebellar neuronal precursors and the formation of medulloblastoma tumors. Furthermore, looking at the localization of the Math1-creERT2::Isl-SmoM2Fl/+ Isl-Pik3caFl/+ tumors it was striking that 13/18 (~72%) had contact to the brainstem. For further analysis it would be interesting if these tumors grow into the brainstem or also develop out of it. These newly developed mouse models can be used for further analysis, e.g. treatment studies.
4.4 LDE225 treatment of Shh-associated mouse models

Inhibition of the Hh pathway is considered as promising therapeutic avenue and small-molecule Hh inhibitors are currently evaluated in early clinical trials for Hh-driven cancers (Low and de Sauvage 2010, Amakye et al. 2013). Hh-driven childhood tumors besides MB are rhabdomyosarcoma (RMS) and rhabdoid tumors (RTs). SMO inhibitors are the leading Hh inhibitors in clinical development, examples are LDE225 and Vismodegib. The remarkable clinical response of a patient with MB to Vismodegib emphasizes the great potential of targeting the Hh pathway in Hh-driven pediatric cancers (Rudin et al. 2009). Based on this proof-of-concept, a phase I/II clinical trial with the SMO inhibitor LDE225 in pediatric advanced solid cancers has been launched (Geoerger et al. 2012). So far, LDE225 turned out to be well-tolerated and clinical responses were observed in patients with documented Hh activation. Recently, a phase III clinical trial with LDE225 in MB has been launched (www.clinicaltrials.gov).

Although a patient with Hh-driven MB initially showed a remarkable response to the SMO inhibitor Vismodegib (Rudin et al. 2009), persistent therapy rapidly led to acquired resistance (Yauch et al. 2009). This indicates that, like other targeted therapies, secondary resistance to SMO inhibitors is a potential hurdle to durable responses in the clinic. In the context of resistance to kinase inhibitors, the elucidation of mechanisms of resistance has enormously accelerated the design of alternative strategies to avoid or overcome resistance. Thus, a better understanding of the molecular mechanisms of resistance against Hh inhibitors is a top priority.

Several other substances for the treatment of MB have already been analysed. Cyclopamine suppresses MB development in Ptch1+/− mice (Sanchez and Ruiz i Altaba 2005). Unfortunately, cyclopamine is not proper for clinical development because of its low oral solubility (Lipinski et al. 2008). More SMO inhibitors are currently being evaluated, including vismodegib (GDC-0449), sonidegib (LDE225), BMS-833923, PF-04449913 and LY2940680, in clinical trials in advanced cancers (Amakye et al. 2013). Among these, vismodegib became the first U.S. Food and Drug Administration (FDA) approved SMO antagonist for the treatment of advanced or metastatic BCC in 2012 (Fellner 2012). Vismodegib significantly lessens the rate of appearance of new BCCs in
patients (Sekulic et al. 2012). Nonetheless, reports showed that most BCCs regrow after vismodegib is stopped (Tang et al. 2012).

GANT61 and HPI-1 are potential therapeutics for MB that target the Hh signalling pathway; both target Gli1 and Gli2 (Lauth et al. 2007, Hyman et al. 2009, Mazumdar et al. 2011). Recent evidence has shown that ATO can inhibit the Hh pathway via mechanisms distinct from that of SMO inhibitors via inhibition of GLI proteins, i.e. by blocking ciliary accumulation of GLI2 and by direct binding and inhibition of GLI1/2 proteins (Kim et al. 2010, Beuchamp et al. 2011). Thus, ATO, a Food and Drug administration (FDA)-approved drug for the treatment of acute promyelocytic leukemia, may offer an alternative treatment option to overcome resistance to SMO inhibitors. Indeed, ATO was recently shown in preclinical models to retain antitumor activity in SMO inhibitor-refractory tumors (Kim et al. 2013). Since concomitant perturbations in additional pathways, which interact with Hh signalling or which become activated as compensatory mechanisms, have been reported in Hh inhibitor-resistant cases, rational drug combinations have been suggested, including PI3K/AKT/mTOR or RAS/MEK/ERK inhibitors or epigenetically acting drugs (Stecca et al. 2007, Buonamici et al. 2010, Wang et al. 2012, Filbin et al. 2013).

LDE225, BMS-833923 and saridegib target SMO (Buonamici et al. 2010, Lee et al. 2012, Lin and Matsui 2012). Here we used LDE225 for the treatment of our Shh-associated mouse models.

Several Smo antagonists including LDE225 are currently being evaluated in clinical trials in patients with advanced solid tumors including medulloblastoma (Tremblay et al. 2009). However, acquired resistance has emerged as a challenge to targeted therapeutics and may limit their anti-cancer efficacy (Engelman and Settleman 2008). Indeed, evidence of resistance to Smo inhibition has recently been reported in a medulloblastoma patient who progressed during therapy with the Smo antagonist GDC0449 (Rudin et al. 2009). Here, mouse strains with an activation of the Shh pathway - *Math1-creERT2::Isl-SmoM2^Fl/+*, *Math1-creERT2::Isl-SmoM2^Fl/+, Isl-Pik3ca^Fl/+*, and *Math1-creERT2::Ptch1^Fl/Fl* - were treated with LDE225 (Figure 21, Figure 23, Figure 24). In both mouse strains with only one mutation (*Math1-creERT2::Isl-SmoM2^Fl/+* and *Math1-creERT2::Ptch1^Fl/Fl*) LDE225 treatment led to a significant better survival rate and to a decrease of proliferation of the tumor.
Finally these mice died of tumor symptoms anyway. Here we suggest that after the 3-week long treatment phase, tumor cells of Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} and Math1-creER\textsuperscript{T2}::Ptc1\textsuperscript{Fl/Ft} mice developed resistance mechanisms. It is conceivable that genetic alterations (mutations, copy-number changes) or changes in gene expression profile appeared after the drug treatment that could explain the acquired resistance. Current evidence suggests that treatment response to SMO inhibitors may only be transient, while resistance rapidly emerges (Kool et al. 2014). Some initially responsive tumors may also acquire secondary resistance to treatment, whereas other patients keep in remission for several years on a single targeted drug. This indicates that, like other targeted therapies, secondary resistance to SMO inhibitors is a potential hurdle to durable responses in the clinic.

Whereas the additional PIK3CA mutation in Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} Isl-Pik3ca\textsuperscript{Fl/+} mice led to an inhibition of the positive effect of LDE225 treatment. The Smo inhibition effect of LDE225 is here not enough. The survival prognosis for LDE225 treated Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} Isl-Pik3ca\textsuperscript{Fl/+} mice does not differ compared to the control group. This upregulation of PI3K signalling could be a mechanism to evade Smo inhibition. Therefore a combination therapy of this Smo inhibitor and an additional PI3K/Tor inhibitor would be useful. A recent study demonstrated that Igf2, through PI3K signalling, potentiates Gli activation induced by low levels of Hh ligand (Riobo et al. 2006). It is possible that under conditions of continuous Hh pathway inhibition, the PI3K pathway compensates for the loss of Hh signalling and thus becomes a major mediator of resistant tumor growth. A combination of the selective PI3K inhibitor BKM120 (also known as Buparlisib), which is already used in clinical trials, and LDE225 constitutes a potential strategy to delay or prevent resistance to Smo antagonists and has important implications for future treatment strategies in medulloblastoma and also other Smo-dependant human cancers.

Interestingly, the earlier treatment start at P20 in Math1-creER\textsuperscript{T2}::Isl-SmoM2\textsuperscript{Fl/+} Isl-Pik3ca\textsuperscript{Fl/+} mice led to a significant better survival prognosis compared to the later treated mice which started at P30 (Figure 23 a). This concludes how important it is to start treatment of tumor as soon as possible. It is conceivable that an even earlier starting point can lead to an even better prognosis.
For future experiments it is planned that the mouse models which were treated with LDE225 (Math1-creER<sup>T2</sup>:\lsl-SmoM2<sup>F1/+</sup>, Math1-creER<sup>T2</sup>:\lsl-SmoM2<sup>F1/+</sup> Isl-Pik3ca<sup>F1/+</sup>, and Math1-creER<sup>T2</sup>:\lsl-Ptch1<sup>F1/F1</sup> mice) will be molecularly analysed in detail. These molecular analyses include whole exome sequencing of tumor DNA and low-coverage WGS of tumor DNA. All samples will be analysed by the mentioned methods before any drug treatment in order to fully characterize these models and identify mutations and copy-number aberrations that could explain any primary drug resistance present in the tumors at the time of biopsy. After drug treatment we will sequence and profile those tumors that became resistant to drug treatment to see whether any genetic alterations (mutations, copy-number changes) or changes in gene expression profiles will appear that could explain the acquired resistance.

Smoothened inhibitors are now available clinically to treat medulloblastoma. However, resistance to these inhibitors rapidly develops thereby limiting their efficacy. The determination of Smoothened crystal structures enables structure-based discovery of new ligands with new chemotypes that will be critical to combat resistance. In a current study 3.2 million available, lead-like molecules against Smoothened were docked, looking for those with high physical complementarity to its structure; this represents the first such campaign against the class Frizzled G-protein coupled receptor family. Twenty-one high-ranking compounds were selected for experimental testing, and four, representing three different chemotypes, were identified to antagonize Smoothened with IC<sub>50</sub> values better than 50 μM. A screen for analogs revealed another six molecules, with IC<sub>50</sub> values in the low micromolar range (Lacroix et al. 2016). Importantly, one of the most active of the new antagonists continued to be efficacious at the D473H mutant of Smoothened, which confers clinical resistance to the antagonist vismodegib in cancer treatment.

In light of recent literature, several considerations are important for future therapeutic development for SHH MB. The first consideration is the variable genetic backgrounds of SHH-MB in infants, children and adults. For example, patients with mutations upstream in the SHH pathway (SHH amplifications, PTCH1 and SMO mutations) in general may respond more favourably to SMO inhibitors, while those with mutations downstream (GLI2 mutations) may be more resistant to treatment (Kool et al. 2014). The second is the development of resistance. An understanding of the radiation and chemotherapy resistance mechanisms of MB is necessary for efficient targeting of medulloblastoma.
Assessing the influence of crosstalk from other signalling pathways will significantly contribute to novel therapeutics for MB with regard to combination therapeutics.
4.5 Perspectives

Currently, the selection of patients with MB that qualify for the clinical trial with the SMO inhibitor LDE225 is based on a five gene signature that indicates Hh pathway activation (Weller et al. 2013). However, this diagnostic test will not be sufficient to identify LDE225-responsive patients, since it cannot distinguish between different aberrations causing Hh activation, e.g. between PTCH1 mutation and GLI1/2 amplification. The innovation of our collaborative project resides in the development of more precise molecular diagnostic tests that not only detect global activation of the Hh pathway, but also allow for the precise definition of the underlying genetic alterations in each individual tumor. This will enable the robust pre-therapeutic stratification of patients for the most suitable Hh inhibitor. In the long-term perspective, these diagnostic assays will be exploited for patient selection in ongoing and future clinical trials with Hh inhibitors for precision oncology.

As just tumors with downstream mutations may be more resistant to treatment, these mutations should be further investigated in mouse studies. One further mouse model which should be developed for future studies carries a GLI2 mutation or the combination of GLI2 amplifications and TP53 mutations, as this was found to occur in children (ages 4-17) (Kool et al. 2014). This model should be characterized in detail and used for treatment studies with ATO and/or GANT61.

In addition to the GEMMs, the antitumor activity of Hh inhibitors in relevant preclinical models including PDX (patient-derived xenograft; human SHH-MB) models could be analysed in parallel with molecular characterization of the corresponding human tumor samples, the results of the project will provide novel insights into which molecular aberrations causing Hh activation are amenable to which type of Hh pathway inhibitor. Depending on the drug responses using single drugs and the molecular alterations identified in these treated tumors, the experiments will be repeated and rational drug combinations will be used to see whether the induced drug resistance can be prevented. The studies in PDX models will be corroborated by our studies in GEMMs with defined aberrations of the Hh signalling pathway. Since more and more Hh inhibitors are being developed to block Hh signalling at distinct levels of the pathway and since more and more molecular lesions are being identified that cause aberrant Hh activation in human cancers, the challenge is to precisely tailor the most suitable Hh inhibitor to the individual patient’s tumor aberrations. For example, patients harboring Smoothened (SMO) mutation will
likely respond to SMO inhibitors, while patients exhibiting GLI2 amplification are expected to benefit from GLI inhibitors. The results of our preclinical studies will be translated into the ongoing and future clinical trials with Hh inhibitors in children. Since the development of rationally targeted cancer therapeutics requires a full understanding of the pharmacokinetic/pharmacodynamics (PK/PD) relationship of the investigational agents, we will also utilize the preclinical models of Hh pathway activation to correlate Hh inhibitor levels in the plasma with pathway inhibition and with antitumor effects. The results of these preclinical PK/PD studies will provide useful PK exposure targets as well as PD endpoints that can be used as gates for further clinical development.

By elucidating primary and acquired resistance mechanisms to SMO inhibitors, which are currently the leading Hh inhibitors under clinical evaluation, the results of the project will pave the way for novel therapeutic strategies to overcome resistance or, alternatively, to avoid resistance by dispatching tumors before they become refractory. For example, GLI1/2 inhibitors such as arsenic trioxide (ATO) may offer an alternative treatment option for cases that are refractory to SMO inhibitors, e.g. due to GLI2 amplification. Another example is the design of combination therapies to concomitantly block compensatory pathways that become activated upon acquired resistance to SMO inhibitors, e.g. by using PI3K/AKT/mTOR inhibitors. Thus, the results of this project will contribute to the development of alternative treatment strategies for Hh inhibitor-resistant patients in many ways, i.e. 1) by discovering the different molecular lesions that cause Hh activation in primary tumor samples, 2) by identifying the mechanism of resistance to Hh inhibitors in PDX models and GEMMs and 3) by evaluating alternative treatment options in refractory mouse models to either circumvent resistance or to avoid the emergence of resistance by pre-emptively treating with a combination. The results of these studies will be translated into the ongoing and future clinical trials with Hh inhibitors in children to design alternative treatment options for patients with primary or acquired resistance to Hh inhibitors. Furthermore, the understanding of common escape mechanisms will help to develop new diagnostics to prescreen patients for the enrolment into clinical trials with Hh inhibitors.

All GEMMs and PDX models will be molecularly characterized by whole exome sequencing, low-coverage WGS (whole genome sequencing), and DNA methylation and gene expression profiling. Tumors that become resistant to Hh pathway inhibition will again be molecularly characterized by sequencing and profiling and data will be compared
to the untreated/vehicle-treated tumors to identify the molecular mechanism(s) of acquired resistance. Based on these molecular findings, rational combination treatments will be used including drugs targeting other pathways co-operating with Hh signalling. All data generated with the mouse models will be compared with molecular data which are currently generated from clinical SHH-MB samples treated with LDE225, the SMO inhibitor that is being used in a phase III clinical trial in Germany embedded in the overall HIT-REZ concept which promotes early clinical trials and therapy optimization studies for children and young adults with recurrent MB and ependymoma. Our increased understanding of primary and secondary resistance mechanism in SHH-MB will form a basis not only for rational combination therapies for primary and relapsed SHH-MB tumors, but also for the development of second-generation Hh inhibitors and for the development of diagnostic tests that can be applied in a clinical setting to predict which patients will be responsive to which drugs.
5 References


References


References


References


6 Appendix

6.1 Supplementary Figures

Figure 25 H&E staining of human (a) and mice (b-f) tumors. Comparison of the morphology of murine tumors with different genotypes to each other and to human SHH-MB. All tumors display classic morphology (small, blue, round cells). Scale bars 20 µm.
Figure 26 Reduced growth of Shh-medulloblastoma after activation of the Wnt-pathway. H&E stains of sagittal cerebellar sections. Activation of the Shh-pathway in *Math1*-positive cerebellar GNPs resulted in a thickened EGL at P0 and the formation of medulloblastoma that were readily detectable at P2 (*Math1-cre::SmoM2^{Ffl/+} genotype). Co-activation of the Wnt/β-catenin-signalling-pathway (*Math1-cre::SmoM2^{Ffl/+}Ctnnb1(ex3)^{Ffl/+} mice) notably reduced growth of medulloblastoma and led to a decreased cerebellar size when compared to *Math1-cre* controls. Adequate cerebellar layering was not seen (see inset with higher magnification). EGL: external granule cell layer, ML: molecular layer, PL: Purkinje cell layer, IGL: internal granule cell layer.
6.2 List of Figures

Figure 1 Sketch of the structure and cytoarchitecture of the cerebellar cortex.......................... 6
Figure 2 Comparison of the various subgroups of medulloblastoma........................................ 10
Figure 3 Hh pathway mutations in 133 sequenced SHH-MB..................................................... 14
Figure 4 The WNT and SHH pathway....................................................................................... 16
Figure 5 Hh signalling pathway............................................................................................... 39
Figure 6 Overview Math1-creERT2::Isl-SmoM2Fli/, Math1-creERT2::Isl-Pik3caFli/, Math1-creERT2::Isl-Pik3caFli/Fli and Math1-creERT2::Isl-SmoM2Fli/+ Isl-Pik3caFli/+ mice................................. 43
Figure 7 Overview Math1-creERT2::Ptc1Fli/, Math1-creERT2::Ptc1Fli/Fli, Math1-creERT2::Ptc1Fli/Fli Isl-Pik3caFli/Fli, Math1-creERT2::Isl-Pik3caFli/+ and Math1-creERT2::Isl-Pik3caFli/+ mice. ............................................................... 46
Figure 10 Anatomical compartments of the murine cerebellum................................................... 52
Figure 11 Sox2 expression in Math1-GFP,Ptc+/− mice............................................................... 52
Figure 12 Representative fluorescent images and H&E stains of MB in Math1-GFP,Ptc+/− mice at different time points.................................................................................................................. 53
Figure 13 (a) MB incidence in Math1-GFP,Ptc+/− mice. (b) Main localization in the cerebellum of Math1-GFP,Ptc+/− mice. (c) Main vertical MB localization. (d) Ventrikel relation of MB................................................................................................................................. 54
Figure 14 (a) RFP stainings indicating equally distributed recombination in granule cells of Math1-creERT2::tdTomato mice. (b) H&E stainings of embryonically and postnatally induced Math1-creERT2::Ish-SmoM2Fli/+ mice. Arrows indicate tumor areas in the vermis and hemisphere. (c) Quantification of the relative tumor area of embryonically and postnatally induced Math1-creERT2::Ish-SmoM2Fli/+ mice in different cerebellar compartments................................................................................................................................. 56
**Figure 15** Cultured cerebellar GNPs from mice of indicated genotypes were transduced with control IRES-GFP virus or Cre-IRES-GFP virus to recombine alleles. Wnt/β-catenin-activation significantly reduced proliferation of both normal GNPs and SmoM2F/Fl transformed GNPs.

**Figure 16** (a-c) Sagittal cerebellar sections. Shh pathway activation in Math1-positive cerebellar GNPs resulted in the formation of medulloblastoma (b). Co-activation of the Wnt/β-catenin signalling pathway [Math1-cre::SmoM2F/Fl/Fl;Ctnnb1(ex3)F/Fl mice] reduced growth of medulloblastoma and led to a decreased cerebellar size (c). (d) Math1-cre::SmoM2F/Fl/Fl;Ctnnb1(ex3)F/Fl mice displayed a significantly prolonged survival.

**Figure 17** Relative quantification of Axin2 and Gli1 in GFP+ SmoM2F/Fl and SmoM2F/Fl/Fl;Ctnnb1(ex3)F/Fl GNPs after Cre-IRES-GFP transduction.

**Figure 18** GSK3β knockout resulted in significantly reduced proliferation of GNPs.

**Figure 19** Lithium chloride (LiCl) reduces medulloblastoma cell viability in vitro.

**Figure 20** Overview over treatment of Shh-associated mouse models.

**Figure 21** (a) Kaplan-Meier survival curve illustrating overall survival of Math1-creERT2::lsl-SmoM2F/Fl mice in LDE225 treatment cohort.

**Figure 22** Comparison of Math1-creERT2::lsl-SmoM2F/Fl and Math1-creERT2::lsl-SmoM2F/Fl lsl-Pik3caF/Fl mice.

**Figure 23** (a) Kaplan-Meier survival curve illustrating overall survival of Math1-creERT2::lsl-SmoM2F/Fl lsl-Pik3caF/Fl mice in LDE225 treatment cohort. (b) Quantification of the fraction of BrdU+ tumor cells.

**Figure 24** (a) Kaplan-Meier survival curve illustrating overall survival of Math1-creERT2::Ptch1F/F mice in LDE225 treatment cohort. (b) Quantification of the fraction of BrdU+ tumor cells.

**Figure 25** H&E staining of human (a) and mice (b-f) tumors. Comparison of the morphology of murine tumors with different genotypes to each other and to human SHH-MB.

**Figure 26** Reduced growth of Shh-medulloblastoma after activation of the Wnt-pathway.
6.3 List of Tables

Table 1 List of primers for genotyping ................................................................. 28
Table 2 PCR conditions for genotyping ............................................................... 29
Table 3 Primary antibodies for immunohistochemistry and immunocytochemistry .... 31
Table 4 Primers for qRT-PCR ........................................................................... 34
Table 5 Conditions for qRT-PCR ...................................................................... 35
### 6.4 List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>(C)GNP</td>
<td>(cerebellar) granule neuron precursor</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>µM</td>
<td>micro-molar</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodesoxyuridine</td>
</tr>
<tr>
<td>C57/Bl6</td>
<td>C57 black 6; common inbred strain of laboratory mouse.</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>cre</td>
<td>cyclization recombinase or causes recombination</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGL</td>
<td>external granular layer</td>
</tr>
<tr>
<td>ETMRs</td>
<td>embryonal tumors with multi-layered rosettes</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-actiated cell sorting</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>Fl/+</td>
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</tr>
<tr>
<td>Fl/FI</td>
<td>homozygous for a „floxed“ allele</td>
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<td>FVB</td>
<td>friend virus B</td>
</tr>
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<td>forward</td>
</tr>
<tr>
<td>Fz</td>
<td>frizzled receptor</td>
</tr>
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<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>GEMM</td>
<td>genetically engineered mouse model</td>
</tr>
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<td>GL261</td>
<td>glioma cell line</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>Hek</td>
<td><em>Human embryonal kidney</em></td>
</tr>
<tr>
<td>HEK 293T</td>
<td>human embryonic kidney cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IGL</td>
<td>inner granular layer</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>IVC</td>
<td>individually ventilated cages</td>
</tr>
<tr>
<td>LCA</td>
<td>large cell/anaplastic</td>
</tr>
<tr>
<td>LiCl</td>
<td>lithium chloride</td>
</tr>
<tr>
<td>loxP</td>
<td>locus of X-over P1</td>
</tr>
<tr>
<td>IsI</td>
<td>lox stop lox</td>
</tr>
<tr>
<td>MB</td>
<td>medulloblastoma</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ML</td>
<td>molecular layer</td>
</tr>
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<td>milliliter</td>
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<tr>
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<td>milli-molar</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NeuN</td>
<td>neuronal nuclei</td>
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<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>P</td>
<td>postnatal day</td>
</tr>
<tr>
<td>PCL</td>
<td>purkinje cell layer</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>PLO</td>
<td>poly-L-ornithine</td>
</tr>
<tr>
<td>PNets</td>
<td>primitive neuroectodermal tumors</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>RCAS</td>
<td>replication competent ASLV long terminal repeat with Splice acceptor</td>
</tr>
<tr>
<td>RL</td>
<td>rhombic lip</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
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<td>RU49</td>
<td>zinc finger transcription factor</td>
</tr>
<tr>
<td>Rv</td>
<td>reverse</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>Shh/SHH</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>S-phase</td>
<td>synthesis phase</td>
</tr>
<tr>
<td>Tam</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TVA</td>
<td>avian retrovirus receptor</td>
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<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>UW473</td>
<td>medulloblastoma cell line</td>
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<tr>
<td>WGS</td>
<td>whole genome sequencing</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>Wnt/WNT</td>
<td>The name Wnt is a portmanteau of int (=proto-oncogene „integration1”) and Wg (Drosophila gene „wingless”) and stands for &quot;Wingless-related integration site&quot;</td>
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6.5 Gene names and definition of gene symbols

Gene symbols written in capital letters indicate human or mouse protein (e.g. MYC)

Gene symbols written in italic capital letters indicate human or mouse gene (e.g. MYC)

<table>
<thead>
<tr>
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<td>AKT</td>
<td>protein kinase B</td>
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<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>ATOH1/MATH1</td>
<td>Protein atonal homolog 1</td>
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<tr>
<td>BMP-4</td>
<td>Bone morphogenetic protein 4</td>
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<tr>
<td>CBP/CREBBP</td>
<td>CREB-binding protein</td>
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<tr>
<td>CD44</td>
<td>CD44 molecule (Indian blood group)</td>
</tr>
<tr>
<td>CDK6</td>
<td>cyclin-dependent kinase 6</td>
</tr>
<tr>
<td>CK1</td>
<td>casein kinase 1</td>
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<tr>
<td>CTNNB1</td>
<td>ß-catenin</td>
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<tr>
<td>DKK1</td>
<td>dickkopf-related protein 1</td>
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<tr>
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<td>dishevelled</td>
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<tr>
<td>EZH2</td>
<td>enhancer of zeste homolog 2</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GLI family zinc finger 1</td>
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<td>GLI2</td>
<td>GLI family zinc finger 2</td>
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<tr>
<td>GLI3</td>
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<td>GSK3ß</td>
<td>Glycogen synthase kinase 3 ß</td>
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<tr>
<td>hGFAP</td>
<td>human glial fibrillary acidic protein</td>
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<td>HOXA2</td>
<td>homeobox A2</td>
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<td>KDM6A</td>
<td>lysine (K)-specific demethylase 6A</td>
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<td>Lac Z</td>
<td>beta-D-galactosidase</td>
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<td>LRP</td>
<td>low-density lipoprotein receptor-related protein</td>
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<td>MLL2/3</td>
<td>myeloid/lymphoid or mixed-lineage leukemia protein 2/3</td>
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<td>MYC (C-MYC)</td>
<td>myelocytomatosis viral oncogene homolog (avian)</td>
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<td>MYCN</td>
<td>myelocytomatosis viral related oncogene, neuroblastoma derived (avian)</td>
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<td>p27-Kip</td>
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<td>PIK3CA</td>
<td>phosphatidylinositol-4,5-bisphosphate3-kinase catalytic subunit alpha</td>
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<td>secreted frizzled-related protein 1</td>
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<td>smoothened, frizzled family receptor</td>
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<td>synuclein alpha interacting protein</td>
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<td>transcription factor/lymphoid enhancer-binding factor</td>
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<tr>
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<td>zinc finger MYM-type containing 3</td>
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6.6 Curriculum Vitae
6.7 List of publications


Engel, N; Neumann, J; Ahlfeld, J; Wefers, A; Merk, D; Ohli, J; Schüller, U. „Canonical Wnt signaling drives tumor-like lesions from Sox2-positive precursors of the murine olfactory epithelium“. PloS One, accepted
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Der letzte Dank gebührt natürlich Matthias, der mich während der Zeit immer unterstützt hat.
Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den ........................................ ...........................................................

(Unterschrift)

Erklärung

Hiermit erkläre ich,*

☐ dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.

☐ dass ich mich anderweitig einer Doktorprüfung ohne Erfolg nicht unterzogen habe.

☐ dass ich mich mit Erfolg der Doktorprüfung im Hauptfach ..................................... und in den Nebenfächern .............................................................. bei der Fakultät für ........................................ der ...........................................................

(Hochschule/Universität) unterzogen habe.

☐ dass ich ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

München, den........................................ ...........................................................

(Unterschrift)

*) Nichtzutreffendes streichen