More than meets the eye: exploring Sonic Hedgehog medulloblastoma from a neurodevelopmental perspective
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General Introduction
Growth Dysregulation:
Bridging Cerebellar Development and Pediatric Medulloblastoma
Chapter 1

**Introduction**

The cerebellum, also known as the "little brain", is located at the posterior end of the brain that serves functions in sensory-motor processing, balance control, reflexes as well as cognitive functions including speech and spatial memory. In humans, cerebellar development starts during embryogenesis at 30 days post-conception and continues up to two years of age. Controlled cerebellar development requires tight regulation of the expression of various developmental genes important in the process, such as transcription factors and morphogenetic signals, ensuing control over core molecular pathways in space and time. However, because of the lengthy period to reach maturity, the cerebellum is vulnerable to growth dysregulation, which may lead to malignant transformation into medulloblastoma, one of the most frequently diagnosed brain tumors in children.

In order to gain more insight into cerebellar developmental defects that promote transformation into medulloblastoma, in the first chapter (Chapter 1) of this thesis, we review cerebellar development by focusing on the formation of cerebellar granule cells, the most abundant cell type of the brain. Their progenitors (e.g., cerebellar granule neuron progenitors or CGNPs) are known to be the cell-of-origin for at least two subtypes of medulloblastoma. We also discuss the biology of the various subtypes of pediatric medulloblastoma with emphasis on the Sonic Hedgehog (SHH) subgroup medulloblastoma. In Chapter 2, we studied the dynamic changes in gene expression in mouse CGNPs during development and shed light on the age-specific onset and pathway mutations that are associated with SHH medulloblastoma. In Chapter 3, we explored the role of transcription factor CREB in CGNP development and medulloblastoma therapy, since we had discovered that CREB phosphorylation status strongly correlates with survival in SHH and Group 3 medulloblastoma. Furthermore, molecular profiling studies done by others have underlined that mutations in genes encoding histone modifier complexes are frequent in medulloblastoma. Therefore, in Chapter 4, we assessed the contribution of one of those genes, the histone modifier KMT2D, to medulloblastoma pathogenesis using a cell line model and shed the light on altered molecular interactions between mutant Kmt2d and other (chromatin) proteins. In the last experimental chapter (Chapter 5), we set out to establish primary pediatric brain tumor cell cultures that faithfully maintain tumor characteristics, which can be employed as model for understanding tumor biology and therapeutic discovery. Finally, we summarize our findings and discuss their implications in Chapter 6.
The Development of Cerebellar Granule Neurons

Summary of Granule Neuron Development
The cerebellum is the part of the brain that is responsible for the integration of sensory information and motor coordination\textsuperscript{2,10}. The development of the cerebellum is quite remarkable and offers an ideal example to study neurogenesis. Due to its excellency in combining cell lineages-specific gene expression with precise environmental signals, the resulting spatio-temporal schedule produces precise neuronal diversity at the cerebellar cortex\textsuperscript{3,6}. In humans, the development of the cerebellum starts from 30 days post-conception and reaches maturity within two years of age\textsuperscript{2,4,5}. However, most of our current knowledge on cerebellum development is derived from studies in mice and rats. The developing murine cerebellum closely resembles that of humans in terms of cell diversity, lamination, circuitry, and basic foliation patterns albeit that they develop much faster\textsuperscript{2,3,11,12}. In mice, gross cerebellar development is almost completed by post-natal day 15 (P15). The mature cerebellar cortex comprises three layers with different neuronal types (Fig. 1). Stellate and basket cells are GABAergic neurons in the molecular layer (ML), underneath which reside GABAergic Purkinje cells in the Purkinje layer (PCL). And in the deepest layer, glutamatergic neurons including the granule cells, unipolar brush cells (UBC), and deep cerebellar nuclei form the internal granular layer (IGL)\textsuperscript{2,13–15}. While most neurons derive from the ventricular epithelium, the majority of cerebellar cells derives from a secondary germinal neuroepithelium known as the external granular layer (EGL) that is populated by cerebellar granule neuron progenitors (CGNPs)\textsuperscript{2,8,16}.

Figure 1. Key developmental stages of mouse cerebellar development from embryonic to early postnatal stage. (a) Specification and migration of RL progenitors consisting of cerebellar granule neuron progenitors (CGNPs) from upper RL (uRL) and Purkinje cell (PC) progenitors in ventricular zone (VZ) at E13.5. (b, c) (Left) Schematics of sagittal sections in developing postnatal day 7 (P7) (b) and near-mature (P14) (c) mouse cerebellum. (Right) Detailed schematics of the cerebellar cortex (boxed regions). (b) At P7, CGNPs undergo massive proliferation forming the outer external granular layer (oEGL, green). Subsequently, CGNPs will exit the cell cycle, forming a specific layer called inner EGL (iEGL, red) containing cells ready for radial migration. Post-mitotic granule cells migrate through the molecular layer (ML) and Purkinje cell layer (PCL, blue), then settle in the internal granular layer (IGL, red). (c) In the almost mature cerebellar cortex at P14, the EGL has mostly disappeared and majority of the granule cells have migrated to the IGL. pia=Pia Mater. Adapted from Martinez et al\textsuperscript{8}.
CGNPs are the most abundant progenitor cell type that ultimately give rise to cerebellar granule cells in the mature cerebellum\textsuperscript{2,17}. In mice, CGNPs are specified and born at E13.5 in the upper rhombic lip (RL), a germinative epithelial region at the interface between the neural tube and roof plate\textsuperscript{11,14,18,19}. Subsequently, CGNPs then proliferate and migrate tangentially over the entire cerebellar anlage to form the EGL\textsuperscript{20,21}. During the early post-natal days, CGNP proliferation reaches their peak proliferation between P4 to P8 at the outer EGL (oEGL). This proliferative spurt causes EGL expansion from a thin layer into a layer of six to eight cells deep\textsuperscript{2,22}. Following the clonal expansion of CGNPs that leads to the extensive foliation of the cerebellum, they will exit the cell cycle as post-mitotic cells and enter a pre-migratory state in the inner EGL (iEGL)\textsuperscript{23–25}. Here, they also start the differentiation program into mature granule neurons, resulting in gradual loss of the EGL. Before starting their inward migration, differentiating granule cells extend their axons parallel to the pial surface thus forming parallel fibers that create synapses with Purkinje cells in the ML, while their cell bodies migrate inward along Bergmann glia to populate the IGL\textsuperscript{26,27}. Finally, post-migratory granule neurons will settle and mature by growing dendrites and creating complex synapse circuitry within the IGL\textsuperscript{2}. In mice, the IGL emerges at P5 and is mostly established by P20, indicating that neurogenesis of granule neurons is completed (Fig. 2).

**Figure 2. Neurogenesis of cerebellar granule neurons.** CGNPs are born and specified in the upper rhombic lip (uRL) at E13.5. Between E15 and E18, they proliferate and migrate tangentially across the surface of the cerebellar anlage, forming a temporary layer called external granular layer (EGL). After birth (broken black line), at around P0 until P8, CGNPs undergo massive proliferation at the outer part of the EGL (oEGL). Subsequently, a proportion of post-mitotic CGNPs populate the inner EGL (iEGL) to start differentiation programs and become cerebellar granule neurons. Shortly before their inward migration, granule cells extend their axons parallel to the pial surface, forming parallel fibers. They then migrate inward along the radial glia and mature in the internal granular layer (IGL) by growing dendrites and creating synapse circuitry. Adapted from Leto et al\textsuperscript{2}.
**Control of CGNP Development**

During the genesis of granule neurons, various factors influence each stage of development. Here, we will discuss the genetic control and molecular regulatory mechanisms, as well as signaling pathways involved in CGNP development, all of which may contribute to medulloblastoma formation when dysregulated (Fig. 3)\(^{28–30}\). In the upper RL, CGNP specification is defined by the expression of basic helix-loop-helix (bHLH) transcription factor, Atoh1 (Math1) which is induced by external signals from bone morphogenetic proteins (BMPs) from the choroid plexus and the roof plate\(^{20,31–33}\). The importance of Math1 in the proliferation of CGNP and other RL-progenitors has been reported in Math1-null mice, where the EGL is absent due to a lack of CGNP proliferation and the offspring dies shortly after birth from respiratory failure\(^{31,34}\). Besides Math1, other studies showed that CGNPs express combination of transcription factors including the zinc finger protein genes Zic1 and Zic3, the paired box gene Pax6, and the homeobox gene Meis1 that also control their development\(^{35,36}\). During the tangential migration phase across the cerebellar surface, CGNPs are still expressing Atoh1 and incidentally also Nestin, a marker for undifferentiated progenitors in a small proportion of cells\(^{18}\).
Perinatally, CGNPs undergo massive proliferation in the oEGL, and multiple mitogenic pathways work together to regulate this process, including the Sonic Hedgehog (SHH) and Notch2 pathways. Sonic Hedgehog (SHH) is a potent diffusible mitogen secreted by Purkinje cells from around E18.5 that becomes a major driver of CGNP proliferation. For example, it was shown that P8 CGNP cultures treated with SHH proliferated faster than control. Additionally, in vivo inhibition of SHH activity resulted in significant reduction of EGL size and the absence of mitotic CGNPs in the oEGL. In addition to its role in CGNP proliferation, SHH is also important for cerebellar foliation patterning by regulating the position and/or size of lobes. The SHH pathway is activated when SHH binds to transmembrane receptor Patched1 (PTCH1) located at the end of primary cilium, an antenna-like structure used for signal transduction. This releases Smoothened (SMO) from its inhibition. Accumulation of active SMO on the ciliary membrane is able to activate transcription factor Gli1 (GLI1) at the tip of the primary cilium, which was previously sequestered by pathway inhibitor Suppressor of Fused (SUFU). Subsequently, Gli1 will translocate to the nucleus and induce expression of cell cycle-related genes, including cyclin D1 (CCND1) and D2 (CCND2), MYCN, as well as other SHH target-genes including PTCH1 and GLI1. Besides the SHH pathway, the Notch2 pathway is also known to stimulate CGNP proliferation as addition of a Notch2 ligand, Jag1, to CGNP cultures significantly promoted proliferation and inhibited granule cell differentiation by sustaining Atoh1 expression.

The iEGL consists of differentiating CGNPs that begin to exit the cell cycle. At this stage, the CGNPs require signals to stop proliferation and initiate differentiation into granule neurons. They induce expression of a major cell cycle inhibitor, p27/Kip1, which acts as an intrinsic factor to exit the cell cycle. It was shown that mice lacking p27/Kip1 increased proliferation at the EGL and exhibited a larger cerebellum. Besides expressing p27/Kip1, post-mitotic CGNPs also had reduced Math1 expression and increased Neurogenic differentiation factor 1 (NeuroD1) and Neuronal nuclei antigen (NeuN) expression, which no longer respond to SHH and thus promote differentiation, respectively. Other environmental signals also provide cues for antagonizing the SHH-proliferative effect, including basic fibroblast growth factor (bFGF) and BMP. bFGF exerts inhibition of proliferation via activation of the mitogen-activated protein kinase (MAPK) pathway, while Bmp4 induced CGNP differentiation by inducing rapid degradation of Math1. Besides, non-canonical activation of the MAPK pathway via ERK1/2 and ERK5 could also be achieved by treatment of CGNPs with WNT3 ligand, which resulted in reduced CGNP proliferation and reduced expression of Atoh1 and SHH-target genes.

Following cell cycle exit, CGNPs receive additional differentiation signals to become mature granule neurons and prepare for inward migration. Initially, they express maturation
marker Tuj1, and interact with specific extracellular matrix (ECM) components, such as Vitronectin and Contactin that lead to the phosphorylation of cyclic-AMP responsive element-binding protein (CREB) to enhance differentiation. They then switch the direction of migration from tangential in the EGL, to radial towards the IGL guided by Semaphorin 6A (Sema6A). Subsequently, they extend their axons to form parallel fibers and start expressing the axonal glycoprotein Tag1 and Doublecortin (DCX), a migratory marker. When radially migrating inward along the Bergmann glia, they move their cell body and release a neuron-glia adhesion junction protein, called Astrotactin, after which they glide along the glial fibers until new adhesions form. Upon arriving at their final destination in the IGL, the granule neurons will first mature by expressing neurotransmitter receptors of both the glutamatergic excitatory and GABAergic inhibitory types. Subsequently, they grow dendrites with a claw-like ending, and finally form synapses with mossy fibers that are essential for generating cerebellar circuitry.

Epigenetic regulation is another important layer of control for proper cerebellar development, involving DNA methylation, chromatin remodeling, and histone modifications. DNA methylation profiling during cerebellar development revealed that the majority of cerebellar genes are hypomethylated, leading to higher gene expression levels compared to other brain regions. This profile was recently confirmed by another genome-wide methylation study in humans, showing that genes related to neurodevelopment, synaptic plasticity, and lipid metabolism are hypomethylated in the human cerebellum. Chromatin remodelers, such as Bmi1 and Chd7 were also shown to play essential roles in cerebellar development. Loss of Bmi1 significantly reduced EGL thickness and caused an arborization defect of basket neurons, while Chd7 deletion in CGNPs resulted in reduced proliferation and cerebellar hypoplasia, leading to a developmental delay. Moreover, histone methylation is among the most studied types of histone modification in brain development and it has been shown that dysregulation of this epigenetic mechanism leads to brain disorders or neurodevelopmental defects. Focusing on histone H3, it is mainly methylated at the lysine (K) residue with mono-, di-, or tri-methylation, or arginine (R) residue with mono- or di-methylation. Methylation at lysine residues (e.g. H3K4, H3K36, H3K79) are associated with gene activation carried out by histone methyltransferase enzymes (KMTs), while demethylation of histone H3 by histone demethylases (KDMs) generally leads to gene repression. Mutations in one of the lysine methyltransferases, KMT2D, resulted in the neurodevelopmental disease termed Kabuki syndrome that is presented by microcephaly, specific facial features, and short stature. Moreover, knockdown of demethylase Kdm5c in rat cerebellar granule neurons significantly reduced their dendritic length. In summary, different layers of molecular control during each step of cerebellar granule cell development are required to guarantee that the cerebellum is fully developed and functioning.
Pediatric Medulloblastoma Biology and Tumorigenesis

An overview of the pediatric medulloblastoma subtypes

Medulloblastoma represents the second most common pediatric brain tumor, accounting for approximately 20% of all childhood brain tumors.\(^{74–76}\) It emerges in the developing cerebellum during the early life from various neuronal progenitor cells.\(^{74–77}\) The most frequent age of onset is in children younger than 10 years old, with about half of the cases diagnosed before the age of 5, although it can also affect teenagers and adolescents.\(^{75,78}\) Diagnosis of medulloblastoma is based on clinical symptoms, brain and spinal imaging, cerebrospinal fluid cytology, as well as histology and molecular analysis.\(^{75}\) The standard treatment involves surgery to remove the tumor, chemotherapy, and irradiation for patients older than 3 years of age (non-infant).\(^{79}\) Medulloblastoma prognosis is dependent on patient age, metastasis at diagnosis, and clinicopathological and molecular features that can be stratified as standard- and high-risk patients. The standard-risk patients, defined as older than 3 years old with complete removal of the tumor and no metastasis at diagnosis, have a relatively good prognosis of 70-85% 5-year overall survival. While patients younger than 3 years old, having suboptimal tumor resection, and/or metastatic disease at diagnosis, are at high-risk with a worse prognosis with less than 70% 5-year overall survival.\(^{78,80}\) Unfortunately, the aggressive treatment procedures often leave medulloblastoma survivors with long-term devastating neurological and cognitive side-effects, thus encouraging clinicians and scientists to tailor improved therapeutics strategies especially for pediatric patients.\(^{79,81,82}\)

In the past two decades, advances in molecular biology have helped dissecting the molecular architecture of pediatric medulloblastoma, which is relevant for improving therapeutic interventions in pediatric patients. In 2012, international consensus was reached on subgrouping medulloblastoma based on transcriptional profiling. The disease now consists of four subgroups (Fig. 4): WNT (Wingless), SHH (Sonic Hedgehog), Group 3, and Group 4.\(^{83}\) Subsequently, further (epi)genomics studies were conducted leading to identification of additional subtypes within the main subgroups.\(^{9,84,85}\) In 2016, the medulloblastoma molecular subgroups were also incorporated into the WHO Classification of Tumors of the Central Nervous System.\(^{76}\)

**Medulloblastoma subgroups**

**WNT subgroup.** WNT medulloblastoma accounts for approximately 10% of all diagnosed cases with a peak in incidences around 10-12 years of age.\(^{86–88}\) The genome-wide gene expression and methylation profiles of the WNT subgroup are in majority homogenous between patients. WNT tumors have a classic histology and infrequent metastatic disease at diagnosis, resulting in a favourable outcome of approximately 95%\(^{75,89,90}\). A somatic activating
Figure 4. Consensus molecular subgroups of pediatric medulloblastoma. There are four molecular subgroups of medulloblastoma: WNT (Wingless), SHH (Sonic Hedgehog), Group 3, and Group 4 with their typical locations and distinct clinicopathological characteristics. RL=rhombic lip; CGNP=cerebellar granule neuron progenitors; GluCN=glutamatergic cerebellar nuclei; UBC=unipolar brush cell; amp=amplification; mut=mutation. Adapted from Northcott et al.  

mutation of CTNNB1, which encodes for β-catenin, and monosomy 6 were discovered in 85-90% and 80-85% of tumors, respectively. The latter two molecular characteristics are therefore considered hallmarks for WNT medulloblastoma. Other recurrent mutations in WNT tumors beside CTNNB1 include DDX3X, SMARCA4, TP53 and KMT2D.  

SHH subgroup. Approximately 28-30% of all medulloblastoma cases belong to the SHH subgroup. SHH medulloblastoma is genetically the best understood subgroup. The majority of SHH medulloblastomas are diagnosed in infants younger than 3 years and adults older than 17 years old, with relatively fewer cases in children. The most common genetic events identified in this subgroup result in constitutive activation of the SHH pathway that include inactivating germline or somatic mutations and deletions of PTCH1 and SUFU, activating mutations of SMO, and amplifications of GLI1, GLI2, or MYCN. Interestingly, these mutations exhibit age-specific differences within the subgroup. Recently, based on DNA methylation and gene expression profiling, SHH medulloblastoma was classified into four subtypes (SHHα, SHHβ, SHHγ and SHHδ) with varying cytogenetics, demographics, and overall survival. There are two infant SHH (iSHH) subtypes, called SHHβ and SHHγ, enriched for germline or somatic SUFU mutations, PTCH1 mutations or chromatin modifying gene (e.g. KMT2D and BCOR) mutations. The SHHα and SHHδ subtypes that correspond to childhood/adult medulloblastomas are enriched for TP53 and TERT promoter as well as SMO mutations, respectively.
**Group 3 and Group 4 subgroups.** The Group 3 and Group 4 tumors constitute about 25% and 35% of all medulloblastoma cases in children, respectively. Being the most aggressive among the subgroups, Group 3 has the worst outcome and up to 50% of tumors has metastasized at diagnosis. Similarly, Group 4 tumors exhibit frequent metastatic spread at diagnosis, but they have a better prognosis than Group 3 tumors. Unlike the WNT and SHH tumors, somatic mutations affecting specific developmental signaling pathways are less common in Group 3 and Group 4 medulloblastoma. However, the most notable genetic alterations in Group 3 tumors is amplification of the *MYC* oncogene, while Group 4 tumors have frequent *MYCN* and cyclin-dependent kinase-6 (*CDK6*) amplification. In addition, a phenomenon known as ‘enhancer hijacking’ that leads to overexpression of GFI1/GFI1B and *PRDM6* are frequently observed in Group 3 and Group 4 tumors, respectively. More recently, DNA methylation profiling studies have suggested to annotate Group 3 and Group 4 medulloblastoma together into subtypes due to intrasubgroup and intersubgroup heterogeneity. This new annotation approach identified eight unique subtypes including subtypes that were comprised exclusively of Group 3 or Group 4 patients, and subtypes that consisted of a combination of both Group 3 and Group 4 patients.

**Developmental origin of medulloblastoma and dysregulated factors**

The intertumoral heterogeneity of medulloblastoma, reflected in each subgroup, suggests that these tumors originate from distinct cellular populations during cerebellum development. It is believed that these cell populations-of-origin undergo transformation due to dysregulation of cell-type specific signaling pathways alongside with subgroup-specific genetic alterations. Various mouse models have been developed to prove this hypothesis and facilitate the search for the cellular origin of the different medulloblastoma subgroups. For example, it was found that WNT tumors originate from lower RL progenitors (Blbp+; embryonic dorsal brainstem (Olig3+) cells that have acquired activating mutations in WNT pathway effectors. On the other hand, the cerebellar progenitors born at the upper RL (Atoh1+; CGNPs) are widely understood to be at the origin of SHH tumors. While the precise cellular origin for Group 3 and Group 4 tumors remains elusive, recent single-cell transcriptional studies of the developing mouse cerebellum and human medulloblastomas has allowed cross-species comparisons between cerebellar cell types and medulloblastoma. This revealed that the putative cells of origin of Group 3 is early embryonic Nestin+ progenitors. The glutamatergic cerebellar nuclei (GluCN) and unipolar brush cells (UBC) were shown to be the probable cell-of-origin for Group 4 medulloblastoma, as this subgroup is strongly regulated by master transcription factors, including LMX1A, EOMES, and LHX2 that are expressed by restricted glutamatergic progenitor populations at the upper RL (Fig. 4).
Among all subgroups, the cellular origin and molecular biology of SHH medulloblastoma is the best understood and most extensively studied. An early mouse model study showed that activation of oncogenic SHH signaling by Ptch heterozygous deletion in granule cell precursors resulted in tumor formation after 3 months of age in about 15% of the mice\textsuperscript{106,107}. SHH pathway activation in neuron progenitors or multi-lineage neural stem cells also resulted in medulloblastoma, but the tumors originating from stem cells only occurred after committing to the neuronal lineage\textsuperscript{108}. Moreover, oncogenic Hedgehog signaling at several other stages of CGNP development was shown to generate comparable medulloblastomas, indicating that acquisition of CGNP identity is essential for SHH medulloblastoma genesis\textsuperscript{109}. Later, these findings were confirmed by recent single-cell transcriptional studies showing that human SHH medulloblastoma tumors represent developmental stages of mouse cerebellar granule cell as shown by similarities in gene expression profiles\textsuperscript{110,113}. Altogether, these results pinpoint that the CGNPs are the cellular origin of SHH medulloblastoma.

The dependency of cerebellar granule cell development on SHH signaling for massive CGNP proliferation after birth has already been established quite some time ago\textsuperscript{23,38,39}. This implicates that deregulation in SHH signaling pathway components caused by genetic lesions can lead to tumorigenic transformation of CGNPs into medulloblastoma (Fig. 5)\textsuperscript{106,109,114}. These lesions include loss-of-function mutations and focal deletions in \textit{PTCH1} and \textit{SUFU}, the negative regulators for the SHH pathway, activating mutations in \textit{SMO}, and amplification of \textit{MYCN} and \textit{GLI2}. Together, these alterations lead to the constitutive, ligand-independent activation of the SHH signaling pathway\textsuperscript{91,94}. Another remarkable mutation in SHH medulloblastoma is the somatic \textit{TP53} mutation, which is enriched among children and adolescents and associated with extremely poor prognosis\textsuperscript{85,115}. In children, this mutation co-occurs frequently with \textit{MYCN} and \textit{GLI2} amplifications but rarely with \textit{PTCH1} mutations, suggesting that the downstream alterations of the SHH signaling pathway might trigger an apoptotic response, which is overcome by \textit{TP53} mutations\textsuperscript{85,91,115}.

Beside direct alterations in SHH signaling pathway components, chromosomal and epigenetic dysregulation are also involved in SHH medulloblastoma. For example, somatic mutation of \textit{TP53} may induce catastrophic genomic rearrangements, known as chromotrisis, which can subsequently lead to aberrant overexpression of SHH ligand caused by chromotrisis-associated fusion of the \textit{DNAJB6} and \textit{SHH} genes\textsuperscript{116,117}. Furthermore, dysregulation of various epigenetic mechanisms, including DNA methylation, histone modifications, and chromatin remodeling also contributes to tumor formation. Long before the genomics era, promoter hypermethylation of the \textit{PTCH1} and \textit{ZIC2} genes was identified in medulloblastoma, suggesting inhibition of negative regulators of the SHH signaling pathway\textsuperscript{118,119}. Overexpression of the oncogene \textit{VAV1} caused by CpG hypomethylation was
found in most human SHH medulloblastoma cases and in mouse models. Interestingly, manipulation of VAV1 levels in medulloblastoma models showed the importance of VAV1 for maintenance of SHH tumors, and its abrogation significantly reduced tumor growth. Moreover, Vav1 regulated CGNP migration in an \textit{ex vivo} model of postnatal cerebellar development\textsuperscript{120}.

Additionally, a high mutational frequency of genes encoding epigenetic regulators was discovered across all medulloblastoma subgroups\textsuperscript{91,121–123}. Among them, somatic mutations affecting histone methyltransferases (e.g., \textit{KMT2C} and \textit{KMT2D}) and demethylases (e.g., \textit{KDM4C} and \textit{KDM4B}), as well as histone acetyltransferases (e.g., \textit{CREBBP}, \textit{EP300}, \textit{KAT6A}, \textit{KAT6B}, and \textit{BRPF1}) and deacetylases (e.g., \textit{BCOR}, \textit{LDB1}, and \textit{GPS2}) were enriched in SHH medulloblastoma\textsuperscript{91,94,121,123}. Histone acetyltransferases play context-dependent roles in SHH medulloblastoma and CGNP development. As an example, embryonic loss of \textit{Crebbp} impairs...
normal cerebellar development due to hypoplasia and disturbed foliation but does not result in medulloblastoma, whereas postnatal Crebbp disruption in CGNPs synergized with the SHH pathway to drive tumor growth\textsuperscript{124}.

Another important group of altered epigenetic regulators in SHH medulloblastoma are the chromatin remodelers, including SMARCA4 (also known as repressing GLI3-mediated expression, and activating GLI1/2-mediated target gene activation\textsuperscript{125}. Similar to the effect of Crebbp loss during cerebellum development, conditional Brg1 deletion leaded to decreased expression of SHH-target genes and reduced CGNP proliferation, which resulted in cerebellar hypoplasia\textsuperscript{126,127}. Curiously, in a SMO-induced medulloblastoma model, reducing or deleting Brg1 in CGNPs impaired tumor initiation, while deletion after tumor formation significantly reduced tumor growth. Conversely, Brg1 was able to promote tumorigenesis via GlI1 and Atoh1 oncogenic activation as well as neuron differentiation, indicating that Brg1 can be required for SHH medulloblastoma development depending on context\textsuperscript{127}. Together, CGNP development is prone to dysregulation in different factors that together lead to SHH medulloblastoma formation.

Resuming, it is clear that during the past decades, our knowledge of medulloblastoma biology has significantly improved. This has moved diagnostics and therapeutic management towards more personalized treatment and targeted therapy, with the aim of reducing patient morbidity and increasing efficacy\textsuperscript{128,129}. It is clear that with this increase in molecular insight into the patient’s tumor, there is also a need for designing preclinical models that accurately model the patient’s tumor. However, while for some pediatric brain tumor types such as SHH medulloblastoma, several preclinical models have been developed (which are not yet exhaustive), for many other brain tumor types including the other medulloblastoma subtypes, only a few or even no appropriate models are available. This highlights an urgent need for improvement.

**Preclinical Models of Pediatric Brain Cancer**

Medulloblastoma is one of many different types of pediatric brain cancer\textsuperscript{74–76}. As a whole, cancer of the central nervous system (CNS) is the second most occurring and the most common solid tumor in children\textsuperscript{74}. Among them, low-grade glioma (LGG) is the most frequently diagnosed pediatric brain tumor (PBT), followed by medulloblastoma (MB), high-grade glioma (HGG), and ependymoma (EPN)\textsuperscript{74,76,129,130}. Based on histopathological classification, all medulloblastoma subgroups belong to WHO grade IV CNS tumors. LGG is generally classified as WHO grade I or II with a favourable outcome, and consists of pilocytic, pilomyxoid, subependymal giant cell, fibrillary, and diffuse astrocytomas\textsuperscript{76,130}. Glioblastoma,
anaplastic astrocytoma, gliomatosis cerebri, and diffuse intrinsic pontine glioma (DIPG) are grouped into HGG and are classified as WHO grade III or IV\textsuperscript{76,129}. EPN is classified based on its location, including posterior fossa, supratentorial, infratentorial and spinal cord\textsuperscript{129}. Recently, a new classification of CNS tumors has been reported that is based on DNA methylation profiling using a comprehensive machine-learning approach. Compared to routine histology diagnostics, this method allows for precise diagnostics, accounting for up to 12\% of new diagnoses when used for re-classification, and thus significantly improving clinical decision-making\textsuperscript{131}.

Historically, PBT treatment consists of several approaches, including surgery, standard irradiation therapy, and conventional chemotherapy, which leave most of the survivors with long-term neurological and cognitive side effects\textsuperscript{132}. Conversely to adult brain tumors, pediatric tumors need highly specialized treatment regimens because the nervous system is still in its developmental window\textsuperscript{129,133}. In line with this, the design of preclinical model for PBT also need specialization and high accuracy in modeling the patient’s tumor, as they are essential for identifying novel therapeutics targeting the patient’s tumor subtype – and not the healthy tissue\textsuperscript{133}. Importantly, these models should also have sufficient translational significance to allow for clinical applications, therefore advancement in the current PBT preclinical models is urgently required\textsuperscript{133}.

There are several kinds of preclinical models for PBT, ranging from simple \textit{in vitro} cell cultures to more complex \textit{in vivo} mouse models. Every model has its advantages and limitations, therefore we propose that applying a strategic combination of models will permit successful clinical translation\textsuperscript{133}. Below, we discuss the available preclinical models for PBT, their benefits and constraints for use, as well as give examples of their applications.

**\textit{In vitro} culture models.** Among the \textit{in vitro} model systems are human or mouse-derived primary cells and cell lines, as well as tumor-derived cell lines. They are routinely used to test biological hypotheses, address mechanistic questions, and prioritise treatment strategies\textsuperscript{130,134}. Besides, they provide valuable platforms for pharmacological high-throughput drug screening in identifying potential novel therapeutics\textsuperscript{135}. Diagnostically, they can be used for high-throughput –omics analyses in assessing specific molecular signatures of PBT to predict outcomes\textsuperscript{128,129,136}.

A primary \textit{in vitro} culture is initiated from cells or tissues harvested from patients or animals. They often contain a mixture of various cell types and tissue components which make them difficult to standardize. This heterogeneity leads to variability and low reproducibility\textsuperscript{137}. However, they still closely resemble their original \textit{in vivo} situation and the degree of similarity can be quantified. Primary cultures become early-passage cultures after
the first passage, where they are maintained under selective pressure in the cell culture media. Once the primary cultures are passaged a number of times, the cells either stop dividing or continue dividing, forming continuous cell lines\textsuperscript{137}. Compared to primary cultures, continuous cell lines are easy to expand and relatively uniform, ensuring lower variability and thus making results more reproducible. Yet, the main hurdles for their utilization are the selection bias, phenotypic and genetic drift\textsuperscript{138}. For example, it was shown that mouse or human SHH medulloblastoma tumors fail to maintain a SHH signaling pathway signature when grown \textit{in vitro}, and fail to generate tumors when the cells were implanted into the mouse brain\textsuperscript{139}.

There are approximately 60 cell lines generated from various types of PBT, including MB, EPN, HGG, and atypical rhabdoid tumor (ATRT)\textsuperscript{140}. The Pediatric Preclinical Testing Program (PPTP) uses a panel of selected PBT cell lines to test drugs for pediatric cancer\textsuperscript{130}. Recently, a standardized method to culture primary PBTs as monolayer cultures in serum-free media was reported, employing special adherent plastic culture vessels and a defined culture medium supplemented with growth factors, EGF and bFGF\textsuperscript{141}. Using this method, several PBTs were able to be propagated including MB, HGG, EPN, and ATRT. Immunofluorescent labelling confirmed phenotypic similarities of these primary monolayer cultures with the corresponding primary tumors. Moreover, they also responded properly when treated using pharmacological inhibitors, suggesting functional utility for preclinical therapeutic assays\textsuperscript{141}.

\textbf{In vivo mouse models}. Mouse models are still considered the gold standard for further preclinical testing of novel therapeutic candidates. Importantly, they have made significant contributions to advancing the understanding of tumor biology, including the mechanism of initiation, progression, and metastasis\textsuperscript{134,142}. Several characteristics are important for accurate PBT modelling in mouse: the model must resemble tumor heterogeneity, have the same developmental origin, and closely mimic the molecular, genetic and epigenetic landscape\textsuperscript{105,143}.

Over the past decades, numerous PBT mouse models have been established, particularly for the most frequently diagnosed brain tumors. Based on the method used, they can be divided into genetically engineered mouse models (GEMM) and (patient derived) xenograft models, the latter including subcutaneous and orthotopic approaches\textsuperscript{105,143,144}. In the past decades, a number of GEMMs for MB and HGG have been established by introducing defined genetic alterations to model tumorigenesis in the cell-of-origin, leaving the native immune system, blood-brain barrier, cell-matrix interactions, and microenvironment intact\textsuperscript{145,146}. Some of the most accurate models used conventional knock-out technology, but also conditional GEMM, RCAS-TV A technique, and the Sleeping beauty transposon system.
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were employed\textsuperscript{134,145,146}. While GEMMs are able to closely recapitulate PBT in mouse, they also have limitations such as the relatively short time taken for tumor development, high cost, unpredictable tumor penetrance, lack of heterogeneity, lack of metastatic disease and variable drug response\textsuperscript{144,146}.

Xenograft models refer to the implantation or injection of primary tumor cells or cell lines sub-cutaneously (\textit{i.e.} under the skin), or orthotopically (into the native tumor site) of immunosuppressed, immunodeficient, or newborn immunonaive mice\textsuperscript{147}. Recently, an increasing number of patient-derived xenograft (PDX)/ patient-derived orthotopic xenograft (PDOX) models from PBT have been generated, providing a better preclinical platform for drug testing and a tool to study tumor biology, as they closely reflect tumor heterogeneity and better represent the human tumor in terms of histology, gene expression, DNA methylation, copy number, and mutational profiles\textsuperscript{148}. Some limitations of PDX models include the choice of animal strain, the use of immunocompromised mice, tumor engraftment, state of the tumor tissue (whether it is treatment-naïve or treated), tumor tissue processing, and ultimately, time and costs\textsuperscript{148,149}. Even though it was previously perceived that PDX models are genomically stable, a recent study reported the presence of karyotype evolution during primary growth and serial passaging in mice, leading to loss of karyotype heterogeneity as well as gain of novel mutations\textsuperscript{150,151}.

Nevertheless, while GEMMs are probably best-suited for studying the origin and progression of PBT and thereby for coming up with novel generalist strategies to target the disease, PDX models might be the best option that we currently have to design personalized treatment. And the latter will become incredibly important, given the huge diversity in (rare) pediatric brain cancers that would be difficult to all develop GEMMs for. If we can establish a high-throughput platform in which the patient’s cells at diagnosis are transplanted to recipient mice in addition to establishing cell cultures, followed by extensive drug screening and molecular profiling, we might have a chance of finding cures for patients that would otherwise not be saved. At the same time, from the collected data new patterns will emerge that will advance our understanding of pediatric brain cancer and complete the picture painted by the genetically engineered PBT models. Altogether, these efforts will lead to an improvement in outcome for the future pediatric brain cancer patient. Additionally, the research collaboration of the Children Oncology Group will enable for better characterization of PDOX models from frequently diagnosed PBT, and these models can be shared with the scientific community to promote the evaluation of potential cancer therapeutics\textsuperscript{152}.
Concluding remarks

During the past decades, our knowledge on cerebellar development and pediatric medulloblastoma has significantly improved and is still advancing. At the cellular level, the cerebellum is the relatively simplest and most powerful model to study neurogenesis. However, its simplicity does not per se associate with complete understanding when it comes to developmental dysregulations leading to the devastating childhood malignancy of medulloblastoma. Therefore, it is essential to integrate the various dysregulated processes at different (molecular) layers during cerebellar development, which possibly promote medulloblastoma tumorigenesis. This thesis is aimed at addressing some of the complex interplay between the molecular players in pediatric SHH medulloblastoma, from the angles of cerebellar development, epigenetic dysregulation, and protein biology. Such knowledge will ultimately lead to improvements in therapy management and care for pediatric patients with SHH medulloblastoma.
References


16. Sotelo, C. Cellular and genetic regulation of the development of the cerebellar system. Progress in


33. Wang, V. Y., Rose, M. F. & Zoghbi, H. Y.


47. Solecki, D. J., Liu, X. L., Tomoda, T.,


108. Yang, Z. J. et al. Medulloblastoma Can Be Initiated by Deletion of Patched in


124. Merk, D. J. et al. Opposing Effects of CREBBP Mutations Govern the Phenotype of Rubinstein-Taybi


