Inter and intra-tumoral heterogeneity as a platform for personalized therapies in medulloblastoma

Marina Danilenko, Steven C. Clifford, Edward C. Schwalbe

PII: S0163-7258(21)00030-9

DOI: https://doi.org/10.1016/j.pharmthera.2021.107828

Reference: JPT 107828

To appear in: *Pharmacology and Therapeutics*

Please cite this article as: M. Danilenko, S.C. Clifford and E.C. Schwalbe, Inter and intra-tumoral heterogeneity as a platform for personalized therapies in medulloblastoma, *Pharmacology and Therapeutics* (2021), https://doi.org/10.1016/j.pharmthera.2021.107828

P&T #23656

Inter and intra-tumoral heterogeneity as a platform for personalized therapies in medulloblastoma

Marina Danilenko[1], Steven C Clifford[1], Edward C Schwalbe[1,2]*

[1] Newcastle University Centre for Cancer, Translational and Clinical Research Institute, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, UK

[2] Department of Applied Sciences, Northumbria University, Newcastle upon Tyne, UK.

*** Corresponding author**

Ed.Schalbe@Northrubria.ac.uk

+44.0191.227.4473

Abstract:

Medulloblastoma is the most common malignant CNS tumor of childhood, affecting ~350 patients/year in the USA. In 2020, most children are cured of their disease, however, survivors are left with life-long late-effects as a consequence of intensive surgery, and application of chemo- and radio-therapy to the developing brain. The major contributor to improvements in patient survival has been the development of risk-stratified treatments derived from a better understanding of the prognostic value of disease biomarkers. The characterization and validation of these biomarkers has engendered a comprehensive understanding of the extensive heterogeneity that exists within the disease, which can occur both between and within tumors (inter- and intra-tumoral heterogeneity, respectively). In this review, we discuss inter-tumoral heterogeneity, describing the early characterization of clinical and histopathological disease heterogeneity, the more recent elucidation of molecular disease subgroups, and the potential for novel therapies based on specific molecular defects. We reflect on the limitations of current approaches when applied to a rare disease. We then review early investigations of intra-tumoral heterogeneity using FISH and immunohistochemical approaches, and focus on the application of next generation sequencing on bulk tumors to elucidate intra-tumoral heterogeneity. Finally, we critically appraise the applications of single-cell sequencing approaches and discuss their potential to drive next biological insights, and for routine clinical application.

Keywords:

Medulloblastoma; intra-tumoral heterogeneity; inter-tumoral heterogeneity

Table of Contents:

Introduction

Medulloblastoma (MB) is the most common malignant CNS tumor of childhood (~350 cases/year in the USA) and is a leading cause of childhood mortality. The primary treatment modality is maximal surgical resection, followed by radiotherapy and/or chemotherapy. Due to improvements in clinical management, neuro-surgical technique, neuro-imaging and the introduction of local and cranio-spinal axis radiotherapy, 5-year survival rates have improved from 2-11% in the 1970s to approximately 75% today (Ellison, Kocak, et al., 2011; Giordana, Schiffer, & Schiffer, 1998; Lannering, et al., 2012). Despite these improvements, there still remain substantial numbers of patients for whom current treatments are ineffective, both in primary and recurrent disease. Moreover, survivors are left with life-long morbidities associated with intensive treatments, in particular a decline in core cognitive abilities (Mulhern, et al., 2005). Now that survival is sustained in the majority of patients, efforts are being made to stratify treatments that balance expected quality of life for survivors whilst maintaining cure rates (Northcott, et al., 2019).

In this review, we detail the extensive clinical, cellular, genetic and functional heterogeneity within MB (Figure 1). Previous biomarker studies have demonstrated how treatment success and failure can be related to biomarkers of inter-tumoral molecular heterogeneity, and the recent application of single-cell sequencing techniques have started to unpick the biological and clinical implications of intra-tumoral heterogeneity within individual MB tumors.

We discuss the history and current understanding of inter-tumoral heterogeneity, summarized in Figure 2, and review single-cell strategies to better delineate intra-tumoral heterogeneity and to characterize new treatment-refractory cell populations. We further speculate how new therapeutic opportunities may be identified by considering each individual MB tumor to be an ensemble of molecularly distinct subclones that reflect a spectrum of dynamic cell states. We also explore why improvements to current conventional therapeutics are plateauing and how an understanding of intra-tumoral heterogeneity can enhance the development of novel treatments to improve patient outcomes, both in terms of survival and long-term quality of life.

Inter-tumoral heterogeneity

Clinical heterogeneity

Inter-tumoral heterogeneity within MB has long been recognized, and a major research focus in the disease is to identify and validate clinico-pathological markers that can be used to predict patient outcomes for treatment individualization. In 1969, Chang et al. defined local tumor extension and tumor dissemination stages (T and M stages respectively) for MB (Chang, Housepian, & Herbert, 1969), and the relationship between distant metastasis and poor clinical outcomes has long been recognized (Zeltzer, et al., 1999). Historically, a subtotal-tumor resection, typically defined as >1.5cm^2 tumor remaining after excision, was associated with poor outcomes (Zeltzer, et al., 1999), however, with improvements in surgery, the difficulty in accurately assessing residual disease, and a better understanding of the molecular heterogeneity within the disease, its prognostic impact is now less clear (E. M. Thompson, Bramall, Herndon, Taylor, & Ramaswamy, 2018; E. M. Thompson, et al., 2016).

Currently, there is a stark treatment difference between 'infant' MB, defined as patients between 3 and 5 years old at diagnosis (dependent on national treatment policies) and childhood MB (non-infant patients <16 years old). Infant patients are treated with protocols that avoid/delay cranio-spinal irradiation, with the aim being to minimize the life-long debilitating late-effects associated with irradiation of a very young brain (Lafay-Cousin, et al., 2009). For childhood patients, current treatment regimens are aimed at ensuring cure and reducing late-effects through risk-adapted chemo- and

radiotherapy, and, looking forwards, the aim is to improve stratification by more precisely applied treatment strategies.

Histopathological inter-tumoral heterogeneity

While initially described as sheets of small, round, blue cells (Rorke, 1983), subsequently, histological variants of MB have been recognized. In current WHO disease definitions (Louis, et al., 2016), the most common histopathological variant is classic MB (70% of cases, characterized by sheets of small, round, blue cells). Desmoplastic/nodular MB (DN; 10-15% of cases) is characterized by reticulin-rich desmoplastic inter-nodular regions and is associated with younger age of diagnosis. Infant desmoplastic/nodular patients are associated with favorable disease outcomes (Hicks, et al., 2020; Rutkowski, et al., 2010). The rarer MB with extensive nodularity (MBEN; 1-2% of cases) is almost exclusively identified in patients under 2 years old (Giangaspero, et al., 1999) and is associated with a favorable survival. Conversely, Large-cell/anaplastic (LCA; 10-15% of cases) disease is characterized by pleomorphic cells and prominent nucleoli and is associated with a worse prognosis (Ellison, Kocak, et al., 2011; Lamont, McManamy, Pearson, Clifford, & Ellison, 2004).

Molecular heterogeneity: Subgroups and subtypes

MB has been a poster child for the genome-wide molecular sub-classification of cancers and their incorporation into routine clinical practice (Clifford, et al., 2015; Northcott, Korshunov, Pfister, & Taylor, 2012; Northcott, et al., 2019). Starting in 2006, studies using genome-wide transcriptomic or DNA-methylation patterns identified varying numbers of distinct molecular subgroups of MB (Cho, et al., 2011; Kool, et al., 2008; Northcott, Korshunov, et al., 2011; M. C. Thompson, et al., 2006); these findings were unified in 2012 to reach an international consensus of four molecularly-defined disease subgroups (M. D. Taylor, et al., 2012). The WNT subgroup (~10% of patients) is defined by activation of the WNT/wingless embryonal signaling pathway; in >90% of WNT tumors, activating mutations of *CTNNB1* stabilize the β-catenin protein, thereby constitutively activating the pathway. The remainder of WNT subgroup tumours are associated with mutations in *APC1* (Goschzik, et al., 2018). This subgroup is characterized by a favorable prognosis (>90% survival) (Clifford, et al., 2006; Ellison, et al., 2005). The SHH subgroup (28% of patients) is defined by activation of the Sonic Hedgehog embryonal signaling pathway and is enriched in infant and adult disease; SHH pathway mutations (e.g. *PTCH1, SUFU, SMO*) characterize the majority of these tumors (Northcott, et al., 2017; Northcott, Hielscher, et al., 2011). Currently, the WHO distinguishes $TP53^{mut}$ and $TP53^{wt}$ variants of SHH disease (Louis, et al., 2016), with the $TP53^{mut}$ variant associated with non-infant disease and very poor disease outcomes (Schwalbe, et al., 2017; Zhukova, et al., 2013). Groups 3 and 4 (27% and 34% of patients respectively (Kool, et al., 2012)) have generic names which reflect the lack of a defined genetic lesion for these closely related subgroups - indeed, the WHO disease definitions class Group 3 and Group 4 as a single, non-WNT/non-SHH entity (Louis, et al., 2016). The definition of the molecular subgroups explained some of the clinico-pathological heterogeneity previously observed disease-wide and led to the concept that MB is an umbrella term for a group of molecularly distinct disease variants. After their initial description, subsequent studies using larger sample numbers and techniques with greater genomic resolution have identified subtypes of subgroups (Cavalli, et al., 2017; Northcott, et al., 2017; Schwalbe, et al., 2017), and, in 2020, subtypes of each of the consensus molecular subgroups have been described (Figure 2). In an international meta-analysis, Sharma et al. described eight DNA-methylation-dependent subtypes of Groups 3/4 (Sharma, et al., 2019), and SHH disease has been split into infant and childhood molecular variants (Kool, et al., 2014; Schwalbe, et al., 2017). More recently, the infant SHH subtype has been further stratified into subtypes I and II (Robinson, et al., 2018), and using combined transcriptomics/methylomics, Cavalli et al. described four molecular subtypes of SHH disease (α, β, γ and δ), mapping to child (α), infant (β, γ) and adult (δ) variants (Cavalli, et al., 2017; Waszak, et al.,

2020); however, as of 2020, consensus definitions and nomenclature of SHH subtypes remain to be defined.

The incorporation of molecular subclassification alongside current clinical biomarkers of MB has enabled the investigation of subgroup-specific, optimized treatment strategies. These can be through reduced therapeutic intensity, which is being investigated for WNT subgroup patients without other high-risk features (clinicaltrials.gov identifiers NCT02066220, NCT01878617, NCT02724579), or through the inhibition of the SHH pathway protein Smoothened in patients with SHH tumors with vismodegib or sonidegib (Li, Song, & Day, 2019; Robinson, et al., 2015).

Current clinical stratification recognizes poor outcomes conferred by amplification of the *MYC* and *MYCN* loci, which are the most commonly amplified oncogenes in MB (Ellison, Kocak, et al., 2011; Northcott, et al., 2019; Ryan, et al., 2012). However, studies of their prognostic impact are inconsistent, suggesting heterogenous disease behavior for these loci, discussed later in this review.

The definition of molecular disease subgroups has enabled the investigation of the impact of subgroup-dependent associations of disease biomarkers, previously defined and characterized on the disease as a whole. Consequently, it has been recognized that while patients with a *MYCN* amplified SHH tumor have dismal outcomes (Schwalbe, et al., 2017), *MYCN* amplified Group 4 patients have similar outcomes to their non-*MYCN* amplified Group 4 counterparts (Goschzik, et al., 2018; Ramaswamy, et al., 2016). The amplification of *MYCN* in SHH disease is associated with LCA histology and mutations in *TP53*. Independently, mutations of *TP53* are a marker of poor-risk and are, in turn, associated with *MYCN* amplifications and LCA histology (Schwalbe, et al., 2017; Zhukova, et al., 2013). LCA histology is also associated with *MYC*-amplified tumors, which are enriched in Group 3 MBs (Schwalbe, et al., 2017). This approach has also been applied to risk-stratification of patient molecular subgroups by cytogenetic markers. In standard-risk Group 3 and Group 4 disease, 2 or more changes among losses of chromosome 8, 11 and gain of chromosome 7 identified a favorable risk group, closely associated with subtypes VI and VII, and with a 100% 5-year survival in the PNET4 clinical trial and near 100% survival in an independent validation cohort (Goschzik, et al., 2018).

Alongside patient groups requiring better treatment options, the application of new investigative techniques is enabling a more refined understanding of the disease. By applying proteomics and phospho-proteomics, it has been shown that the molecular subgroups show highly heterogenous post-transcriptional modifications, and that transcriptionally similar tumors can vary at the post-transcriptional and post-translational level, enabling a better functional understanding of disease biology; Forget et al. described how aberrant ERBB4-SRC signaling is a hallmark, and putative target for novel therapies, in Group 4 MBs (Archer, et al., 2018; Forget, et al., 2018).

Inter-tumoral heterogeneity of immune markers

Immunotherapy is being rapidly developed for targeting tumor-specific cell-surface antigens (Riley, June, Langer, & Mitchell, 2019; Terry, et al., 2020). However, unlike other paediatric brain tumours (e.g. glioblastomas, astrocytomas and ependymomas), MBs typically do not have large amounts of immune cell infiltrates and, surprisingly, their immune landscape strongly resembles healthy brain tissue (Griesinger, et al., 2013). Nevertheless, a 2020 study by Donovan et al. identified three cell-surface antigens, EPHA2, HER2 and interleukin 13 receptor α2, that were expressed by MB cells and absent on the cell surface of normal brain cells. They reported that subsequent administration of CAR T cells targeting these epitopes was a highly efficient therapy in xenograft mouse models of Group 3 metastatic MB (Donovan, et al., 2020).

A 2020 study by Grabovska et al. applied a deconvolution approach, methylCIBERSORT, to DNA methylation microarray data from 2,325 MBs to explore inter-tumoral immune variation and the

composition of the tumour immune microenvironment (TIME). They reported a significant immune heterogeneity among medulloblastoma subgroups and subtypes (Grabovska, et al., 2020). The most frequently infiltrated non-cancer cell types across all MB subgroups were CD8T, B-cells, and eosinophils. Remarkably, the proportions of immune cell types differed significantly between the four consensus molecular subgroups, and *MYC* amplification in Group 3 tumours was associated with a distinct TIME defined by significantly increased proportions of CD8T and B cells. Consensus clustering of MB immune cell profiles identified four immune clusters. Immune cluster membership 'cut across' the consensus molecular subgroups, and was associated with specific clinico-molecular features, offering potential for a more refined understanding of clinical behaviour when TIME as well as consensus molecular subgroup is considered.

Future approaches

The molecular era has enabled the subclassification of the disease and, from this, groups of patients with favorable outcomes (e.g. WNT subgroup patients) have been identified and are eligible for therapy de-escalation in current trials. There are also groups of patients (*MYC* amplified Group 3 tumors; *TP53* mutated/*MYCN* amplified SHH tumors) for whom 5-year survival rates are unacceptably low (*i.e.* < 20%). Since conventional treatments are failing in these patients, new treatment approaches or philosophies are needed, however the potential for dramatic survival improvements using new combinations of conventional chemo- and radiotherapy is limited.

Molecular sub-divisions within a rare disease such as MB makes the investigation of the clinical and biological relevance of these subgroups and subtypes very challenging, and it is envisaged that future studies will necessitate international co-operation to collect sample cohorts of sufficient size. For example, the recent international meta-analysis that defined eight subtypes of Group 3 and Group 4 MB included 1,501 patient samples, representing >2 years' worth of total MB patients from USA and Europe combined (Sharma, et al., 2019). Consequently, using these approaches, this level of disease stratification may have been taken to its furthest logical extreme; however, a unified multi-omic study (NGS, DNA methylation, transcriptome, proteomics, metabolomics), applied to a cohort size > 1000, has yet to be published. Additionally, the NGS techniques currently applied to bulk tumors have reached their maximal (i.e. genome-wide, per base calls) genomic resolution. New approaches are therefore needed to continue to improve patient outcomes.

Future improvements to treatments may involve novel therapeutics (Hill, et al., 2015; Kumar, et al., 2017) for patients with poor prognoses. Related to this are the very poor survival rates following disease relapse; once patients have received cranio-spinal irradiation, there are currently no other effective treatment options and consequently, long-term survival following disease relapse is between 5 and 12% (Johnston, et al., 2018; Pizer & Clifford, 2009).

Intra-tumoral heterogeneity

Not every genetic lesion, be it a driver or a passenger alteration, will necessarily be present in every cell of a MB tumor. In addition to inter-tumoral variation between MBs from different patients, evidence has emerged for the presence and clinical impact of diversity within MB tumors - this diversity is termed intra-tumoral heterogeneity (McBride, Rida, & Aneja, 2015; Park, Gönen, Kim, Michor, & Polyak, 2010; Yates & Campbell, 2012; Ye, et al., 2009).

Early studies of intra-tumoral heterogeneity

Numerous bulk tumor studies have reported intra-tumoral heterogeneity of key MB biomarkers (e.g. LCA histology, *MYC(N)* amplifications, *CTNNB1* and *TP53* mutations) through the application of

conventional wet-lab techniques, such as histopathological assays, immunohistochemistry and FISH (Ellison, 2010; Hill, et al., 2015; Korshunov, et al., 2012; Pietsch & Haberler, 2016; Ryan, et al., 2012; X. Wang, et al., 2013).

Histopathological intra-tumoral heterogeneity

The distinction of MB into the histopathological subtypes recognized by the WHO can be problematic, since MBs often display a complex admixture of subtypes within the same tumor. Two typical examples of morphological intra-tumoral heterogeneity are mixed-classic and LC/A as well as mixed-DN and MBEN histological subtypes (Ellison, 2010; Kool, et al., 2012; Rausch, et al., 2012) (Figure 3.1A). The extent and relevance of heterogeneity, defined by their distinct spatial histology, is not yet clearly defined for such MBs. It is also unknown whether these heterogeneous types have distinct molecular correlates. Understanding the clinical, pathologic and prognostic role of intra-tumoral heterogeneity in MB tissue morphology and its link with molecular intra-tumoral heterogeneity in MB tumorigenesis is an area for future studies.

Intra-tumoral heterogeneity of protein biomarkers by immunohistochemistry

Alongside histopathological subtype, the immunohistochemical (IHC) detection of biomarkers is routinely applied in MB diagnostics to distinguish MB from other brain tumor entities of a similar location and histopathology (e.g. ependymoma) as well as to estimate intra-tumoral heterogeneity (Chiang & Ellison, 2017). Numerous IHC studies have demonstrated that MBs show highly heterogeneous patterns of key protein biomarkers among cells within the same tumor (Figure 3.1B) (de Haas, et al., 2006; Eberhart, Tihan, & Burger, 2000; Ellison, Dalton, et al., 2011; Hill, et al., 2015). Nuclear accumulation of β-catenin protein in at least 10% of MB cells is a clinically significant intra-tumoral heterogeneity cut-off score applied in standard molecular testing of MB (Pietsch & Haberler, 2016; Silva, et al., 2013). Ellison et al. tested 235 MBs and demonstrated a strong heterogeneity of GAB1 protein expression among inter- and intra-nodular compartments of DN tumors (Ellison, Dalton, et al., 2011), while a study by Hill et al. reported heterogenous *TP53* protein expression in 29 relapsed MBs (Hill, et al., 2015). Although IHC is useful for the characterization of spatial intra-tumoral heterogeneity for selected biomarkers, its application to intra-tumoral heterogeneity-focused studies is limited. Since conventional IHC assays are optimized to detect and quantify the expression of a single marker of interest per tissue section, the characterization of a specific biomarker by intra-tumoral heterogeneity in the context of other molecular alterations is challenging. Furthermore, initial tissue preservation conditions, tissue pre-processing for optimized antigen retrieval and individual sectioning skills can strongly affect the nature and reproducibility of results from IHC. Finally, being dependent on successful antibody assays, IHC requires numerous optimization and control steps as well as an accurate scoring system to avoid false positive/negative intra-tumoral heterogeneity counts/scores**.**

Intra-tumoral heterogeneity of chromosomal defects by FISH

Fluorescence in-situ hybridization (FISH) is a commonly used method to identify tumor-specific chromosomal aberrations and is a gold-standard tool for detection of aneuploidy and specific oncogene amplifications in MB diagnostics and research. In 2012, applying interphase FISH, a study by Ryan et al. observed highly variable cellular gene amplification patterns underlying *MYC* copy number amplifications in MB patient biopsies (Ellison, Kocak, et al., 2011; Ryan, et al., 2012). Furthermore, intra-tumoral heterogeneity in the amplification of *MYCN* has been reported in multiple MB studies (Figure 3.1C) (Aldosari, et al., 2002; Ellison, Kocak, et al., 2011; Lamont, et al., 2004; Ryan, et al., 2012). The inherent intra-tumoral heterogeneity for *MYC/N* has necessitated empirically-defined guidelines for

scoring of their amplification for clinical application. For example, a *MYC/N* amplification cut-off of ≥5% of cells in 200 non-overlapping nuclei is applied in current SIOP-PNET5-MB clinical trial (Pietsch & Haberler, 2016). Even though conventional FISH techniques provide a clear assessment of intra-tumoral heterogeneity status for candidate single CNAs, FISH protocols are labor intensive (involving a number of fixation, incubation and washing steps) and since each probe can only typically target one chromosomal aberration, the quantitative intra-tumoral heterogeneity assessment of the cytogenetic landscape of chromosomal alterations requires application of more advanced technologies (Rodriguez-Mateos, Azevedo, Almeida, & Pamme, 2020).

Intra-tumoral heterogeneity in the molecular era of Next Generation Sequencing (NGS)

NGS studies have greatly improved an understanding of inter-tumoral heterogeneity and identified key sets of cancer driving events in MB (Hovestadt, et al., 2019; Northcott, et al., 2017; Ocasio, et al., 2019; A. M. Taylor, et al., 2018; Zhang, et al., 2019). Since the emergence of NGS, MB studies of intra-tumoral heterogeneity have followed two major approaches, focusing on the spatial and inferred heterogeneity within bulk tumors and the characterization of intra-tumoral heterogeneity at the single-cell level.

Spatial analysis of intra-tumoral heterogeneity in bulk tumors by multi-regional sampling

In some types of cancer, e.g. non-small cell lung cancer and clear cell renal cell carcinoma, subclonal changes are spatially distinct (de Bruin, et al., 2014; Gerlinger, et al., 2014). Thus, clinical diagnoses based on single tumor biopsies might be dependent upon the sampling site, potentially leading to patients receiving sub-optimal therapies based in part on incorrect molecular and histological diagnostics. To address the clinical significance of spatial heterogeneity in MB, Morrissy et al. performed a study assessing multiple biopsies derived from the same MB tumors (Morrissy, et al., 2017) (Figure 3.2). Importantly, none of the assessed tumors were uniformly homogeneous or heterogeneous: tumors showed spatial homogeneity at the transcriptome level and distinct patterns of spatial mutational heterogeneity. Divergent clonality of key driver mutations was detected across multiple biopsies, and the authors suggested that spatial heterogeneity of MB should be considered when targeting single mutations therapeutically. The study also presented some evidence of homogenous expression of antigen-coding genes within multiple biopsies from 9 primary MBs (Morrissy, et al., 2017); the intra-tumoral heterogeneity of immune biomarkers in MB remains an area of research that requires further exploration.

Currently, MBs can be reliably assigned to a molecular subgroup from just a single biopsy based on their transcriptome (Northcott, Korshunov, et al., 2011; Northcott, Shih, et al., 2012; Schwalbe, et al., 2011) or, more commonly, their methylome (Northcott, et al., 2017; Schwalbe, et al., 2017; Sharma, et al., 2019), however, in their paper, Morrissy et al. suggested that to identify 80% of mutations in any tumor, at least 5 biopsies need to be tested, with at least 4 biopsies required to detect only 50% of alterations in highly heterogeneous tumors (Morrissy, et al., 2017).

Caveats of bulk NGS approaches

The suitability of using NGS sequencing of bulk tumors to elucidate intra-tumoral heterogeneity can be problematic. When bioinformatically inferring intra-tumoral heterogeneity and clonal evolution from a single sequenced biopsy, spatial heterogeneity is not assessed. To catalogue a more complete landscape of heterogeneity, a number of biopsies need to be bulk sequenced and subsequently compared, which is expensive and often not practical given the availability and size of tumor material recovered from surgery (Morrissy, et al., 2017). Moreover, bulk sequencing data does not reflect the number of cells and the size of a cancer specimen. Thus, in downstream analyses, samples with different physical dimensions might show different patterns of clonal evolution that is modulated in part by their size and cellularity, with more cellular specimens showing an older ancestral history in contrast to samples with a lower cell content. Another challenge faced by bulk tumor sequencing is contamination by stromal cells

(low tumor purity), which reduces the frequency of cancer alleles in bulk sequencing data (Turajlic, Sottoriva, Graham, & Swanton, 2019). Limitations in allelic copy number inference (the number of genome copies at a given site) and SNV assignment (number of allelic copies for a given mutation) may result in a misleading clonal profile when derived from bulk sequencing data.

Intra-tumoral heterogeneity of medulloblastoma by single-cell sequencing

Single-cell sequencing is an emerging alternative to bulk sequencing. In contrast to bulk sequencing, single-cell approaches allow comparison of the frequency of aberrations among multiple individual cells within the same tumor (McGranahan & Swanton, 2015). Single-cell sequencing has been applied to investigate intra-tumoral heterogeneity in many cancers (Fu, et al., 2020; Izar, et al., 2020; Qian, et al., 2020). Remarkably, a single-cell study of genomic intra-tumoral heterogeneity of breast cancer by Wang et al. suggested that any two single cells originating from the same tumor would have a different genomic profile (Y. Wang, et al., 2014).

Developmental origin of medulloblastoma cells by single-cell transcriptomics

Prior to the emergence of single-cell sequencing, genotype-to-cell-type associations have been studied, with proposed cells of origin being extra-cerebellar cells from the dorsal brainstem for WNT MBs (Gibson, et al., 2010) and cerebellar granule neuron progenitors (GNPs) (Oliver, et al., 2005; Yang, et al., 2008) for SHH MBs. Transcriptome analysis of individual cells allows the characterization of stochastic mRNA fluctuations in cells of the same type, to further distinguish diverse cell types, as well as to uncover the phenotypic and morphological properties of cells within a tumor (Raj & van Oudenaarden, 2008). Several recent studies have applied single-cell RNA sequencing approaches to characterize the transcriptome of single MB cells (Figure 3.3A). Hovestadt et al. investigated the cellular origin of twenty-five MBs belonging to four molecular subgroups at a single-cell level, and, based on gene expression differences, characterized tissue-specific cellular and developmental states of MB. Tumors belonging to WNT, SHH and Group 3 subgroups were shown to consist of undifferentiated and differentiated neuronal-like malignant populations. Group 4 tumors were composed of differentiated neuronal-like neoplastic cells, with evidence for the cell of origin of Group 4 tumors being unipolar brush cells and glutamatergic cerebellar nuclei (Hovestadt, et al., 2019). A contemporaneous study by Vladoiu et al. confirmed that MB consists of mixed cellular populations with divergent differentiation trajectories. In this study, the SHH MB subgroup was shown to transcriptionally mirror the granule cell hierarchy, and Group3 tumors to be similar to Nestin-positive stem cells, while Group4 MBs resembled unipolar brush cells (Vladoiu, et al., 2019). The clinical implications of understanding intra-tumoral heterogeneity in the context of SHH subgroup MB have been explored using single-cell RNA sequencing in two further studies. Ocasio et al. assessed cellular diversity in response to treatment by the SHH-pathway - Smoothened inhibitor, vismodegib. Treatment with this drug increased cellular differentiation and reduced the proliferative capacity of SHH tumors in transgenic, MB-prone mice (Ocasio, et al., 2019). Zhang et al. identified that *OLIG2*-expressing glial progenitors were highly enriched in therapy-resistant and relapsing MBs (Zhang, et al., 2019).

Clonal structure and temporal intra-tumoral heterogeneity by single-cell genome sequencing

Genomic alterations in cancer comprise a fundamental resource of cellular diversity (Junker & van Oudenaarden, 2014). Intra-tumoral heterogeneity acts as a substrate for genomic tumor evolution, whereby sequential acquisition of alterations generates genetically related subpopulations of cells within the tumor. Multiple studies (e.g. on glioblastoma, breast, kidney and ovarian cancers) are

beginning to characterize the temporal process of acquisition of mutational lesions (Casasent, et al., 2018; McGranahan & Swanton, 2015; McPherson, et al., 2016; J. Wang, et al., 2016; Xu, et al., 2012). A study by Gerstung et al. inferred clonal evolution patterns in 38 cancer types (totaling 2,061 tumors, including 146 MBs) using genomic data obtained from bulk tumor samples. This study reported that chromosomal aneuploidy is an early event in MB development, with loss of chromosome 17q as well as mutations in *DDX3X* and *PTCH1* being key early driver events (Gerstung, et al., 2020). However, given this work was performed on bulk sequencing data, it only provided a broad and inferred overview of the evolutionary history of MB, neither reflecting intra-tumoral heterogeneity nor considering these changes in a subgroup-specific manner. The application of single-cell WGS to tumors allows aneuploidy detection and phylogeny to be directly inferred without deconvolution, and outperforms bulk sequencing for more precise branching and tracking the phylogenetic relationships between mutations with similar allelic frequency (Malikic, Jahn, Kuipers, Sahinalp, & Beerenwinkel, 2019). Thus, single-cell genome sequencing offers clear potential for a more accurate estimate of MB clonal substructure compared to bulk sequencing in the future (Figure 3.3B).

Spatial intra-tumoral heterogeneity at single-cell resolution

Understanding the relationship among single-cells and their relative locations within a tumor is crucial for understanding MB pathology. Emerging spatial transcriptomics approaches offer a near-single-cell or even a sub-cellular resolution. For instance, the application of spatially-barcoded arrays and subsequent data deconvolution allowed assessment of genetic heterogeneity up to a single-cell level in ~2,200 regions of melanoma tissue (Thrane, Eriksson, Maaskola, Hansson, & Lundeberg, 2018) as well as ~6,750 tissue domains of prostate cancer tumors (Berglund, et al., 2018). Berglund et al. identified distinct expression profiles for the normal tissue components, namely stroma, glands and immune cells were identified and compared with spatial transcriptional profiles of cancer cells. High-resolution spatial transcriptomic profiling of serial MB sections on a single-cell level is a promising technique to uncover a whole 3D landscape of MB tissue, helping to identify new therapeutic biomarkers (Figure 3.3C).

Medulloblastoma subgrouping and single-cell DNA methylation profiling

Extensive epigenomic profiling of 110,294 nuclei, combining the information on DNA methylation, chromatin contact and chromatin accessibility was performed by Liu et al, generating an atlas of DNA methylation of the mouse brain at single-cell resolution (Liu, et al., 2020). Studying epigenetic intra-tumoral heterogeneity at a single-cell level is crucial for understanding MB tumorigenesis, since the currently understood molecular subtypes of subgroups of MB have been defined by DNA methylation patterns. Recent evidence indicates the presence of intra-tumoral heterogeneity of methylation status in Group 3 and Group 4 MB samples, with single tumors having mixed methylation patterns consistent with Group 3 and Group 4 subgroups (Łastowska, et al., 2018). International consensus studies have been undertaken to define heterogeneity in Group 3 and Group 4 MB according to molecular subtypes, assigning more specific subgroup and subtype definitions (Northcott, et al., 2017; Northcott, et al., 2019; Sharma, et al., 2019). Single-cell assessment of genome-wide DNA methylation would be a suitable technique to further improve MB subtyping by providing a catalogue of intra-tumoral heterogeneity and allowing cell-specific subtyping to be performed.

Limitations of single-cell sequencing

Due to the inherently limited yield of nucleic acids from a single cell, amplification methods have to be applied to provide a sufficient amount of material for sequencing. Amplification bias, however, can result in a poor coverage, high background noise as well as allelic dropout (Junker & van Oudenaarden, 2014; Zahn, et al., 2017). While single-cell genomics to assess aneuploidy and/or mutation detection can be performed on DNA extracted from fresh-frozen or formalin-fixed, paraffin embedded specimens, most published protocols for single-cell transcriptomics are dependent upon high-quality fresh or frozen

tumour tissue, which therefore precludes analysis of archival specimens. Single-cell sequencing is a developing and currently expensive field of research which continues to rapidly evolve. The application of automated techniques and robotics will enable greater numbers of processed cells with more uniform sample handling. There is much scope for the statistical development of methodologies to reduce noise and amplification-free approaches are being actively developed. Due to costly techniques and reagents, all published single-cell MB studies described in this review were performed on relatively small tumor cohorts of up to 25 MBs. As costs decrease, techniques will become more accessible, and we believe that intra-tumoral heterogeneity assessment will be invaluable for the elucidation of the fundamental biological processes underpinning tumorigenesis and for the clinical investigation of these findings.

Clinical significance and application of intra-tumoral heterogeneity in medulloblastoma

Once intra-tumoral heterogeneity biomarkers have been identified, their clinical relevance and potential to improve diagnostic accuracy should be investigated. Furthermore, understanding the significant longitudinal steps of clonal evolution in each MB subgroup, and their timing in tumorigenesis might allow 'evolution-aware' disease management, with potential for improved clinical outcomes and a lower incidence of disease recurrence. Clinical trials of the future should also take into account that driver events might be heterogeneous, while different subclones might be targeted by specific inhibitors. For instance, the DARWIN trial (Deciphering Anti-tumor Response With Intratumor Heterogeneity (clinicaltrials.gov identifier NCT02183883) investigated whether progression-free survival of non-small lung cell cancer could be improved by targeting the clonally dominant tumor driver, and the clinical behavior of the same driver in its subclonal stage of evolution.

Summary

In this review, we have discussed the history and current understanding of inter- and intra-tumoral heterogeneity within MB. Through an improved understanding of heterogeneity, a more refined disease sub-classification has driven better patient outcomes, through biomarker identification, and subsequent application of molecularly-determined, risk-adapted therapies. To effectively treat patients for whom current therapies are unsuccessful, it will be important to develop more refined treatment personalization for patients by adopting validated findings from large, multi-omic bulk tumor studies allied to novel biological insights from single-cell intra-tumoral heterogeneity studies. However, balanced against the potential of outcomes to be improved by new disease understanding is the requirement to develop and employ routinely-applicable, low-cost clinical assays for key disease biomarkers arising from these studies.

Acknowledgements:

This review was supported by Cancer Research UK (grants C8464/A13457 and C8464/A23391), and as part of the INSTINCT network (funded by The Brain Tumour Charity, Children with Cancer UK and Great Ormond Street Hospital Children's Charity).

Conflict of Interest Statement:

The authors declare no conflicts of interest.

Figure Legends:

Figure 1: Medulloblastoma is a heterogeneous disease. Schematic illustrates an interplay of intra- and inter-tumoral heterogeneity of medulloblastoma and highlights the clinical significance of heterogeneity for patients.

Figure 2: Timeline of inter-tumoral heterogeneity discovery in medulloblastoma. Key advances in the understanding of clinical, molecular and proteomic inter-tumoral heterogeneity are shown.

Figure 3: Timeline representing the development of key approaches for the investigation of intra-tumoral heterogeneity within medulloblastoma: **1** – Microscopy as a tool to uncover intra-tumoral heterogeneity: **1A** – Histopathological heterogeneity with mixed patterns of DN and MBEN histology within a frozen tissue section stained by H&E; **1B** – TP53 protein expression heterogeneity among single cells of MB FFPE tissue section assessed by immunohistochemical targeting of the anti-TP53 antigen; **1C** – Heterogeneity in a *MYC* copy number profile assessed by *MYC*-specific iFISH probes. **2** – Representation of the bulk NGS approaches applied to research intra-tumoral heterogeneity based on the study of Morrissy et al. (2017). **3** – Representation of the single-cell technology advances used to assess intra-tumoral heterogeneity by single-cell sequencing: **3A** – Example t-SNE plot illustrating the abundance of single-cell clusters with variable functions/distinct cellular origins, using single-cell transcriptomics analyses; **3B** – Schematic dendrogram representing the subclonal structure of medulloblastoma on a single-cell level; **3C** – Illustration of a spatial transcriptomics experiment with a frozen MB tissue section placed onto a barcoded slide to explore a spatial landscape of MB at a subcellular resolution.

References:

Aldosari, N., Bigner, S. H., Burger, P. C., Becker, L., Kepner, J. L., Friedman, H. S., & McLendon, R. E. (2002). MYCC and MYCN oncogene amplification in medulloblastoma. A fluorescence in situ hybridization study on paraffin sections from the Children's Oncology Group. *Arch Pathol Lab Med, 126*, 540-544.

Archer, T. C., Ehrenberger, T., Mundt, F., Gold, M. P., Krug, K., Mah, C. K., Mahoney, E. L., Daniel, C. J., LeNail, A., Ramamoorthy, D., Mertins, P., Mani, D. R., Zhang, H., Gillette, M. A., Clauser, K., Noble, M., Tang, L. C., Pierre-Francois, J., Silterra, J., Jensen, J., Tamayo, P., Korshunov, A., Pfister, S. M., Kool, M., Northcott, P. A., Sears, R. C., Lipton, J. O., Carr, S. A., Mesirov, J. P., Pomeroy, S. L., & Fraenkel, E. (2018). Proteomics, Post-translational Modifications, and Integrative Analyses Reveal Molecular Heterogeneity within Medulloblastoma Subgroups. *Cancer Cell, 34*, 396-410 e398.

Berglund, E., Maaskola, J., Schultz, N., Friedrich, S., Marklund, M., Bergenstråhle, J., Tarish, F., Tanoglidi, A., Vickovic, S., Larsson, L., Salmén, F., Ogris, C., Wallenborg, K., Lagergren, J., Ståhl, P., Sonnhammer, E., Helleday, T., & Lundeberg, J. (2018). Spatial maps of prostate cancer transcriptomes reveal an unexplored landscape of heterogeneity. *Nat Commun, 9*, 2419.

Casasent, A. K., Schalck, A., Gao, R., Sei, E., Long, A., Pangburn, W., Casasent, T., Meric-Bernstam, F., Edgerton, M. E., & Navin, N. E. (2018). Multiclonal Invasion in Breast Tumors Identified by Topographic Single Cell Sequencing. *Cell, 172*, 205-217.e212.

Cavalli, F. M. G., Remke, M., Rampasek, L., Peacock, J., Shih, D. J. H., Luu, B., Garzia, L., Torchia, J., Nor, C., Morrissy, A. S., Agnihotri, S., Thompson, Y. Y., Kuzan-Fischer, C. M., Farooq, H., Isaev, K., Daniels, C., Cho, B. K., Kim, S. K., Wang, K. C., Lee, J. Y., Grajkowska, W. A., Perek-Polnik, M., Vasiljevic, A., Faure-Conter, C., Jouvet, A., Giannini, C., Nageswara Rao, A. A., Li, K. K. W., Ng, H. K., Eberhart, C. G., Pollack, I. F., Hamilton, R. L., Gillespie, G. Y., Olson, J. M., Leary, S., Weiss, W. A., Lach, B., Chambless, L. B., Thompson, R. C., Cooper, M. K., Vibhakar, R., Hauser, P., van Veelen, M. C., Kros, J. M., French, P. J., Ra, Y. S., Kumabe, T., López-Aguilar, E., Zitterbart, K., Sterba, J., Finocchiaro, G., Massimino, M., Van Meir, E. G., Osuka, S., Shofuda, T., Klekner, A., Zollo, M., Leonard, J. R., Rubin, J. B., Jabado, N., Albrecht, S., Mora, J., Van Meter, T. E., Jung, S., Moore, A. S., Hallahan, A. R., Chan, J. A., Tirapelli, D. P. C., Carlotti, C. G., Fouladi, M., Pimentel, J., Faria, C. C., Saad, A. G., Massimi, L., Liau, L. M., Wheeler, H., Nakamura, H., Elbabaa, S. K., Perezpeña-Diazconti, M., Chico Ponce de León, F., Robinson, S., Zapotocky, M., Lassaletta, A., Huang, A., Hawkins, C. E., Tabori, U., Bouffet, E., Bartels, U., Dirks, P. B., Rutka, J. T., Bader, G. D., Reimand, J., Goldenberg, A., Ramaswamy, V., & Taylor, M. D. (2017). Intertumoral Heterogeneity within Medulloblastoma Subgroups. *Cancer Cell, 31*, 737-754.e736.

Chang, C. H., Housepian, E. M., & Herbert, C., Jr. (1969). An operative staging system and a megavoltage radiotherapeutic technic for cerebellar medulloblastomas. *Radiology, 93*, 1351-1359.

Chiang, J. C., & Ellison, D. W. (2017). Molecular pathology of paediatric central nervous system tumours. *J Pathol, 241*, 159-172.

Cho, Y. J., Tsherniak, A., Tamayo, P., Santagata, S., Ligon, A., Greulich, H., Berhoukim, R., Amani, V., Goumnerova, L., Eberhart, C. G., Lau, C. C., Olson, J. M., Gilbertson, R. J., Gajjar, A., Delattre, O., Kool, M., Ligon, K., Meyerson, M., Mesirov, J. P., & Pomeroy, S. L. (2011). Integrative genomic analysis of medulloblastoma identifies a molecular subgroup that drives poor clinical outcome. *J Clin Oncol, 29*, 1424-1430.

Clifford, S. C., Lannering, B., Schwalbe, E. C., Hicks, D., O'Toole, K., Nicholson, S. L., Goschzik, T., Zur Muhlen, A., Figarella-Branger, D., Doz, F., Rutkowski, S., Gustafsson, G., Pietsch, T., & Group, S. I.-E. P. (2015). Biomarker-driven stratification of disease-risk in non-metastatic medulloblastoma: Results from the multi-center HIT-SIOP-PNET4 clinical trial. *Oncotarget, 6*, 38827-38839.

Clifford, S. C., Lusher, M. E., Lindsey, J. C., Langdon, J. A., Gilbertson, R. J., Straughton, D., & Ellison, D. W. (2006). Wnt/Wingless pathway activation and chromosome 6 loss characterize a distinct molecular sub-group of medulloblastomas associated with a favorable prognosis. *Cell Cycle, 5*, 2666-2670.

de Bruin, E. C., McGranahan, N., Mitter, R., Salm, M., Wedge, D. C., Yates, L., Jamal-Hanjani, M., Shafi, S., Murugaesu, N., Rowan, A. J., Grönroos, E., Muhammad, M. A., Horswell, S., Gerlinger, M., Varela, I., Jones, D., Marshall, J., Voet, T., Van Loo, P., Rassl, D. M., Rintoul, R. C., Janes, S. M., Lee, S. M., Forster, M., Ahmad, T., Lawrence, D., Falzon, M., Capitanio, A., Harkins, T. T., Lee, C. C., Tom, W., Teefe, E., Chen, S. C., Begum, S., Rabinowitz, A., Phillimore, B., Spencer-Dene, B., Stamp, G., Szallasi, Z., Matthews, N., Stewart, A., Campbell, P., & Swanton, C. (2014). Spatial and temporal diversity in genomic instability processes defines lung cancer evolution. *Science, 346*, 251-256.

de Haas, T., Oussoren, E., Grajkowska, W., Perek-Polnik, M., Popovic, M., Zadravec-Zaletel, L., Perera, M., Corte, G., Wirths, O., van Sluis, P., Pietsch, T., Troost, D., Baas, F., Versteeg, R., & Kool, M. (2006). OTX1 and OTX2 expression correlates with the clinicopathologic classification of medulloblastomas. *J Neuropathol Exp Neurol, 65*, 176-186.

Donovan, L. K., Delaidelli, A., Joseph, S. K., Bielamowicz, K., Fousek, K., Holgado, B. L., Manno, A., Srikanthan, D., Gad, A. Z., Van Ommeren, R., Przelicki, D., Richman, C., Ramaswamy, V., Daniels, C., Pallota, J. G., Douglas, T., Joynt, A. C. M., Haapasalo, J., Nor, C., Vladoiu, M. C., Kuzan-Fischer, C. M., Garzia, L., Mack, S. C., Varadharajan, S., Baker, M. L., Hendrikse, L., Ly, M., Kharas, K., Balin, P., Wu, X., Qin, L., Huang, N., Stucklin, A. G., Morrissy, A. S., Cavalli, F. M. G., Luu, B., Suarez, R., De Antonellis, P., Michealraj, A., Rastan, A., Hegde, M., Komosa, M., Sirbu, O., Kumar, S. A., Abdullaev, Z., Faria, C. C., Yip, S., Hukin, J., Tabori, U., Hawkins, C., Aldape, K., Daugaard, M., Maris, J. M., Sorensen, P. H., Ahmed, N., & Taylor, M. D. (2020). Locoregional delivery of CAR T cells to the cerebrospinal fluid for treatment of metastatic medulloblastoma and ependymoma. *Nat Med, 26*, 720-731.

Eberhart, C. G., Tihan, T., & Burger, P. C. (2000). Nuclear localization and mutation of beta-catenin in medulloblastomas. *J Neuropathol Exp Neurol, 59*, 333-337.

Ellison, D. W. (2010). Childhood medulloblastoma: novel approaches to the classification of a heterogeneous disease. *Acta Neuropathol, 120*, 305-316.

Ellison, D. W., Dalton, J., Kocak, M., Nicholson, S. L., Fraga, C., Neale, G., Kenney, A. M., Brat, D. J., Perry, A., Yong, W. H., Taylor, R. E., Bailey, S., Clifford, S. C., & Gilbertson, R. J. (2011). Medulloblastoma: clinicopathological correlates of SHH, WNT, and non-SHH/WNT molecular subgroups. *Acta Neuropathologica, 121*, 381-396.

Ellison, D. W., Kocak, M., Dalton, J., Megahed, H., Lusher, M. E., Ryan, S. L., Zhao, W., Nicholson, S. L., Taylor, R. E., Bailey, S., & Clifford, S. C. (2011). Definition of disease-risk stratification groups in childhood medulloblastoma using combined clinical, pathologic, and molecular variables. *J Clin Oncol, 29*, 1400-1407.

Ellison, D. W., Onilude, O. E., Lindsey, J. C., Lusher, M. E., Weston, C. L., Taylor, R. E., Pearson, A. D., Clifford, S. C., & United Kingdom Children's Cancer Study Group Brain Tumour, C. (2005). beta-Catenin status predicts a favorable outcome in childhood medulloblastoma: the United Kingdom Children's Cancer Study Group Brain Tumour Committee. *J Clin Oncol, 23*, 7951-7957.

Forget, A., Martignetti, L., Puget, S., Calzone, L., Brabetz, S., Picard, D., Montagud, A., Liva, S., Sta, A., Dingli, F., Arras, G., Rivera, J., Loew, D., Besnard, A., Lacombe, J., Pages, M., Varlet, P., Dufour, C., Yu, H., Mercier, A. L., Indersie, E., Chivet, A., Leboucher, S., Sieber, L., Beccaria, K., Gombert, M., Meyer, F. D., Qin, N., Bartl, J., Chavez, L., Okonechnikov, K., Sharma, T., Thatikonda, V., Bourdeaut, F., Pouponnot, C., Ramaswamy, V., Korshunov, A., Borkhardt, A., Reifenberger, G., Poullet, P., Taylor, M. D., Kool, M., Pfister, S. M., Kawauchi, D., Barillot, E., Remke, M., & Ayrault, O. (2018). Aberrant ERBB4-SRC Signaling as a Hallmark of Group 4 Medulloblastoma Revealed by Integrative Phosphoproteomic Profiling. *Cancer Cell, 34*, 379-395 e377.

Fu, W., Wang, W., Li, H., Jiao, Y., Huo, R., Yan, Z., Wang, J., Wang, S., Wang, J., Chen, D., Cao, Y., & Zhao, J. (2020). Single-Cell Atlas Reveals Complexity of the Immunosuppressive Microenvironment of Initial and Recurrent Glioblastoma. *Front Immunol, 11*, 835.

Gerlinger, M., Horswell, S., Larkin, J., Rowan, A. J., Salm, M. P., Varela, I., Fisher, R., McGranahan, N., Matthews, N., Santos, C. R., Martinez, P., Phillimore, B., Begum, S., Rabinowitz, A., Spencer-Dene, B., Gulati, S., Bates, P. A., Stamp, G., Pickering, L., Gore, M., Nicol, D. L., Hazell, S., Futreal, P. A., Stewart, A. & Swanton, C. (2014). Genomic architecture and evolution of clear cell renal cell carcinomas defined by multiregion sequencing. *Nat Genet, 46*, 225-233.

Gerstung, M., Jolly, C., Leshchiner, I., Dentro, S. C., Gonzalez, S., Rosebrock, D., Mitchell, T. J., Rubanova, Y., Anur, P., Yu, K., Tarabichi, M., Deshwar, A., Wintersinger, J., Kleinheinz, K., Vázquez-García, I., Haase, K., Jerman, L., Sengupta, S., Macintyre, G., Malikic, S., Donmez, N., Livitz, D. G., Cmero, M., Demeulemeester, J., Schumacher, S., Fan, Y., Yao, X., Lee, J., Schlesner, M., Boutros, P. C., Bowtell, D. D., Zhu, H., Getz, G., Imielinski, M., Beroukhim, R., Sahinalp, S. C., Ji, Y., Peifer, M., Markowetz, F., Mustonen, V., Yuan, K., Wang, W., Morris, Q. D., Spellman, P. T., Wedge, D. C., & Van Loo, P. (2020). The evolutionary history of 2,658 cancers. *Nature, 578*, 122-128.

Giangaspero, F., Perilongo, G., Fondelli, M. P., Brisigotti, M., Carollo, C., Burnelli, R., Burger, P. C., & Garre, M. L. (1999). Medulloblastoma with extensive nodularity: a variant with favorable prognosis. *J Neurosurg, 91*, 971-977.

Gibson, P., Tong, Y., Robinson, G., Thompson, M. C., Currle, D. S., Eden, C., Kranenburg, T. A., Hogg, T., Poppleton, H., Martin, J., Finkelstein, D., Pounds, S., Weiss, A., Patay, Z., Scoggins, M., Ogg, R., Pei, Y., Yang, Z. J., Brun, S., Lee, Y., Zindy, F., Lindsey, J. C., Taketo, M. M., Boop, F. A., Sanford, R. A., Gajjar, A., Clifford, S. C., Roussel, M. F., McKinnon, P. J., Gutmann, D. H., Ellison, D. W., Wechsler-Reya, R., & Gilbertson, R. J. (2010). Subtypes of medulloblastoma have distinct developmental origins. *Nature, 468*, 1095-1099.

Giordana, M. T., Schiffer, P., & Schiffer, D. (1998). Prognostic factors in medulloblastoma. *Childs Nerv Syst, 14*, 256-262.

Goschzik, T., Schwalbe, E. C., Hicks, D., Smith, A., Zur Muehlen, A., Figarella-Branger, D., Doz, F., Rutkowski, S., Lannering, B., Pietsch, T., & Clifford, S. C. (2018). Prognostic effect of whole chromosomal aberration signatures in standard-risk, non-WNT/non-SHH medulloblastoma: a retrospective, molecular analysis of the HIT-SIOP PNET 4 trial. *Lancet Oncol, 19*, 1602-1616.

Grabovska, Y., Mackay, A., O'Hare, P., Crosier, S., Finetti, M., Schwalbe, E. C., Pickles, J. C., Fairchild, A. R., Avery, A., Cockle, J., Hill, R., Lindsey, J., Hicks, D., Kristiansen, M., Chalker, J., Anderson, J., Hargrave, D., Jacques, T. S., Straathof, K., Bailey, S., Jones, C., Clifford, S. C., & Williamson, D. (2020). Pediatric pan-central nervous system tumor analysis of immune-cell infiltration identifies correlates of antitumor immunity. *Nat Commun, 11*, 4324.

Griesinger, A. M., Birks, D. K., Donson, A. M., Amani, V., Hoffman, L. M., Waziri, A., Wang, M., Handler, M. H., & Foreman, N. K. (2013). Characterization of distinct immunophenotypes across pediatric brain tumor types. *J Immunol, 191*, 4880-4888.

Hicks, D., Rafiee, G., Schwalbe, E. C., Howell, C. I., Lindsey, J. C., Hill, R. M., Smith, A. J., Adidharma, P., Steel, C., Richardson, S., Pease, L., Danilenko, M., Crosier, S., Joshi, A., Wharton, S. B., Jacques, T. S., Pizer, B., Michalski, A., Williamson, D., Bailey, S., & Clifford, S. C. (2020). The molecular landscape and associated clinical experience in infant medulloblastoma: prognostic significance of second-generation subtypes. *Neuropathol Appl Neurobiol*.

Hill, R. M., Kuijper, S., Lindsey, J. C., Petrie, K., Schwalbe, E. C., Barker, K., Boult, J. K., Williamson, D., Ahmad, Z., Hallsworth, A., Ryan, S. L., Poon, E., Robinson, S. P., Ruddle, R., Raynaud, F. I., Howell, L., Kwok, C., Joshi, A., Nicholson, S. L., Crosier, S., Ellison, D. W., Wharton, S. B., Robson, K., Michalski, A., Hargrave, D., Jacques, T. S., Pizer, B., Bailey, S., Swartling, F. J., Weiss, W. A., Chesler, L., & Clifford, S. C. (2015). Combined MYC and P53 defects emerge at medulloblastoma relapse and define rapidly progressive, therapeutically targetable disease. *Cancer Cell, 27*, 72-84.

Hovestadt, V., Smith, K. S., Bihannic, L., Filbin, M. G., Shaw, M. L., Baumgartner, A., DeWitt, J. C., Groves, A., Mayr, L., Weisman, H. R., Richman, A. R., Shore, M. E., Goumnerova, L., Rosencrance, C., Carter, R. A., Phoenix, T. N., Hadley, J. L., Tong, Y., Houston, J., Ashmun, R. A., DeCuypere, M., Sharma, T., Flasch, D., Sil kov, A., Ligon, K. L., Pomeroy, S. L., Rivera, M. N., Rozenblatt-Rosen, O., Rusert, J. M., Wechsler-Reya, R. J., Li, X. N., Peyrl, A., Gojo, J., Kirchhofer, D., Lötsch, D., Czech, T., Dorfer, C., Haberler, C., Geyeregger, R., Halfmann, A., Gawad, C., Easton, J., Pfister, S. M., Regev, A., Gajjar, A., Orr, B. A., Slavc, I., Robinson, G. W., Bernstein, B. E., Suvà, M. L., & Northcott, P. A. (2019). Resolving medulloblastoma cellular architecture by single-cell genomics. *Nature, 572*, 74-79.

Izar, B., Tirosh, I., Stover, E. H., Wakiro, I., Cuoco, M. S., Alter, I., Rodman, C., Leeson, R., Su, M. J., Shah, P., Iwanicki, M., Walker, S. R., Kanodia, A., Melms, J. C., Mei, S., Lin, J. R., Porter, C. B. M., Slyper, M., Waldman, J., Jerby-Arnon, L., Ashenberg, O., Brinker, T. J., Mills, C., Rogava, M., Vigneau, S., Sorger, P. K., Garraway, L. A., Konstantinopoulos, P. A., Liu, J. F., Matulonis, U., Johnson, B. E., Rozenblatt-Rosen, O., Rotem, A., & Regev, A. (2020). A single-cell landscape of high-grade serous ovarian cancer. *Nat Med*.

Johnston, D. L., Keene, D., Strother, D., Taneva, M., Lafay-Cousin, L., Fryer, C., Scheinemann, K., Carret, A. S., Fleming, A., Afzal, S., Wilson, B., Bowes, L., Zelcer, S., Mpofu, C., Silva, M., Larouche, V., Brossard, J., & Bouffet, E. (2018). Survival Following Tumor Recurrence in Children With Medulloblastoma. *J Pediatr Hematol Oncol, 40*, e159-e163.

Junker, J. P., & van Oudenaarden, A. (2014). Every cell is special: genome-wide studies add a new dimension to single-cell biology. *Cell, 157*, 8-11.

Kool, M., Jones, D. T., Jager, N., Northcott, P. A., Pugh, T. J., Hovestadt, V., Piro, R. M., Esparza, L. A., Markant, S. L., Remke, M., Milde, T., Bourdeaut, F., Ryzhova, M., Sturm, D., Pfaff, E., Stark, S., Hutter, S., Seker-Cin, H., Johann, P., Bender, S., Schmidt, C., Rausch, T., Shih, D., Reimand, J., Sieber, L., Wittmann, A., Linke, L., Witt, H., Weber, U. D., Zapatka, M., Konig, R., Beroukhim, R., Bergthold, G., van Sluis, P., Volckmann, R., Koster, J., Versteeg, R., Schmidt, S., Wolf, S., Lawerenz, C., Bartholomae, C. C., von Kalle, C., Unterberg, A., Herold-Mende, C., Hofer, S., Kulozik, A. E., von Deimling, A., Scheurlen, W., Felsberg, J., Reifenberger, G., Hasselblatt, M., Crawford, J. R., Grant, G. A., Jabado, N., Perry, A., Cowdrey, C., Croul, S., Zadeh, G., Korbel, J. O., Doz, F., Delattre, O., Bader, G. D., McCabe, M. G., Collins, V. P., Kieran, M. W., Cho, Y. J., Pomeroy, S. L., Witt, O., Brors, B., Taylor, M. D., Schuller, U., Korshunov, A., Eils, R., Wechsler-Reya, R. J., Lichter, P., Pfister,

S. M., & Project, I. P. T. (2014). Genome sequencing of SHH medulloblastoma predicts genotype-related response to smoothened inhibition. *Cancer Cell, 25*, 393-405.

Kool, M., Korshunov, A., Remke, M., Jones, D. T., Schlanstein, M., Northcott, P. A., Cho, Y. J., Koster, J., Schouten-van Meeteren, A., van Vuurden, D., Clifford, S. C., Pietsch, T., von Bueren, A. O., Rutkowski, S., McCabe, M., Collins, V. P., Backlund, M. L., Haberler, C., Bourdeaut, F., Delattre, O., Doz, F., Ellison, D. W., Gilbertson, R. J., Pomeroy, S. L., Taylor, M. D., Lichter, P., & Pfister, S. M. (2012). Molecular subgroups of medulloblastoma: an international meta-analysis of transcriptome, genetic aberrations, and clinical data of WNT, SHH, Group 3, and Group 4 medulloblastomas. *Acta Neuropathol, 123*, 473-484.

Kool, M., Koster, J., Bunt, J., Hasselt, N. E., Lakeman, A., van Sluis, P., Troost, D., Meeteren, N. S., Caron, H. N., Cloos, J., Mrsic, A., Ylstra, B., Grajkowska, W., Hartmann, W., Pietsch, T., Ellison, D., Clifford, S. C., & Versteeg, R. (2008). Integrated genomics identifies five medulloblastoma subtypes with distinct genetic profiles, pathway signatures and clinicopathological features. *PLoS One, 3*, e3088.

Korshunov, A., Remke, M., Kool, M., Hielscher, T., Northcott, P. A., Williamson, D., Pfaff, E., Witt, H., Jones, D. T., Ryzhova, M., Cho, Y. J., Wittmann, A., Benner, A., Weiss, W. A., von Deimling, A., Scheurlen, W., Kulozik, A. E., Clifford, S. C., Peter Collins, V., Westermann, F., Taylor, M. D., Lichter, P., & Pfister, S. M. (2012). Biological and clinical heterogeneity of MYCN-amplified medulloblastoma. *Acta Neuropathol, 123*, 515-527.

Kumar, V., Kumar, V., McGuire, T., Coulter, D. W., Sharp, J. G., & Mahato, R. I. (2017). Challenges and Recent Advances in Medulloblastoma Therapy. *Trends Pharmacol Sci, 38*, 1061-1084.

Lafay-Cousin, L., Bouffet, E., Hawkins, C., Amid, A., Huang, A., & Mabbott, D. J. (2009). Impact of radiation avoidance on survival and neurocognitive outcome in infant medulloblastoma. *Curr Oncol, 16*, 21-28.

Lamont, J. M., McManamy, C. S., Pearson, A. D., Clifford, S. C., & Ellison, D. W. (2004). Combined histopathological and molecular cytogenetic stratification of medulloblastoma patients. *Clin Cancer Res, 10*, 5482-5493.

Lannering, B., Rutkowski, S., Doz, F., Pizer, B., Gustafsson, G., Navajas, A., Massimino, M., Reddingius, R., Benesch, M., Carrie, C., Taylor, R., Gandola, L., Bjork-Eriksson, T., Giralt, J., Oldenburger, F., Pietsch, T., Figarella-Branger, D., Robson, K., Forni, M., Clifford, S. C., Warmuth-Metz, M., von Hoff, K., Faldum, A., Mosseri, V., & Kortmann, R. (2012). Hyperfractionated versus conventional radiotherapy followed by chemotherapy in standard-risk medulloblastoma: results from the randomized multicenter HIT-SIOP PNET 4 trial. *J Clin Oncol, 30*, 3187-3193.

Łastowska, M., Trubicka, J., Niemira, M., Paczkowska-Abdulsalam, M., Karkucińska-Więckowska, A., Kaleta, M., Drogosiewicz, M., Perek-Polnik, M., Krętowski, A., Cukrowska, B., Grajkowska, W., Dembowska-Bagińska, B., & Matyja, E. (2018). Medulloblastoma with transitional features between Group 3 and Group 4 is associated with good prognosis. *J Neurooncol, 138*, 231-240.

Li, Y., Song, Q., & Day, B. W. (2019). Phase I and phase II sonidegib and vismodegib clinical trials for the treatment of paediatric and adult MB patients: a systemic review and meta-analysis. *Acta Neuropathol Commun, 7*, 123.

Liu, H., Zhou, J., Tian, W., Luo, C., Bartlett, A., Aldridge, A., Lucero, J., Osteen, J. K., Nery, J. R., Chen, H., Rivkin, A., Castanon, R. G., Clock, B., Li, Y. E., Hou, X., Poirion, O. B., Preissl, S., O'Connor, C., Boggeman, L., Fitzpatrick, C., Nunn, M., Mukamel, E. A., Zhang, Z., Callaway, E. M., Ren, B., Dixon, J. R., Behrens, M. M., & Ecker, J. R. (2020). DNA Methylation Atlas of the Mouse Brain at Single-Cell Resolution. *bioRxiv*, 2020.2004.2030.069377.

Louis, D. N., Perry, A., Reifenberger, G., von Deimling, A., Figarella-Branger, D., Cavenee, W. K., Ohgaki, H., Wiestler, O. D., Kleihues, P., & Ellison, D. W. (2016). The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol, 131*, 803-820.

Malikic, S., Jahn, K., Kuipers, J., Sahinalp, S. C., & Beerenwinkel, N. (2019). Integrative inference of subclonal tumour evolution from single-cell and bulk sequencing data. *Nat Commun, 10*, 2750.

McBride, M., Rida, P. C., & Aneja, R. (2015). Turning the headlights on novel cancer biomarkers: Inspection of mechanics underlying intratumor heterogeneity. *Mol Aspects Med, 45*, 3-13.

McGranahan, N., & Swanton, C. (2015). Biological and therapeutic impact of intratumor heterogeneity in cancer evolution. *Cancer Cell, 27*, 15-26.

McPherson, A., Roth, A., Laks, E., Masud, T., Bashashati, A., Zhang, A. W., Ha, G., Biele, J., Yap, D., Wan, A., Prentice, L. M., Khattra, J., Smith, M. A., Nielsen, C. B., Mullaly, S. C., Kalloger, S., Karnezis, A., Shumansky, K., Siu, C., Rosner, J., Chan, H. L., Ho, J., Melnyk, N., Senz, J., Yang, W., Moore, R., Mungall, A. J., Marra, M. A., Bouchard-Côté, A., Gilks, C. B., Huntsman, D. G., McAlpine, J. N., Aparicio, S., & Shah, S. P. (2016). Divergent modes of clonal spread and intraperitoneal mixing in high-grade serous ovarian cancer. *Nat Genet, 48*, 758-767.

Morrissy, A. S., Cavalli, F. M. G., Remke, M., Ramaswamy, V., Shih, D. J. H., Holgado, B. L., Farooq, H., Donovan, L. K., Garzia, L., Agnihotri, S., Kiehna, E. N., Mercier, E., Mayoh, C., Papillon-Cavanagh, S., Nikbakht, H., Gayden, T., Torchia, J., Picard, D., Merino, D. M., Vladoiu, M., Luu, B., Wu, X., Daniels, C., Thorswell, S., Thompson, Y. Y., Hovestadt, V., Northcott, P. A., Jones, D. T. W., Peacock, J., Wang, X., Mack, S. C., Reimand, J., Albrecht, S., Fontebasso, A. M., Thiessen, N., Li, Y., Schein, J. E., Lee, D., Carlsen, R., Mayo, M., Tse, K., Tam, A., Dhalla, N., Ally, A., Chuah, E., Cheng, Y., Plettner, P., Li, H. I., Corbett, R. D., Wong, T., Long, W., Loukides, J., Buczkowicz, P., Hawkins, C. E., Tabori, U., Rood, B. R., Myseros, J. S., Packer, R. J., Korshunov, A., Lichter, P., Kool, M., Pfister, S. M., Schuller, U., Dirks, P., Huang, A., Bouffet, E., Rutka, J. T., Bader, G. D., Swanton, C., Ma, Y., Moore, R. A., Mungall, A. J., Majewski, J., Jones, S. J. M., Das, S., Malkin, D., Jabado, N., Marra, M. A., & Taylor, M. D. (2017). Spatial heterogeneity in medulloblastoma. *Nat Genet, 49*, 780-788.

Mulhern, R. K., Palmer, S. L., Merchant, T. E., Wallace, D., Kocak, M., Brouwers, P., Krull, K., Chintagumpala, M., Stargatt, R., Ashley, D. M., Tyc, V. L., Kun, L., Boyett, J., & Gajjar, A. (2005). Neurocognitive consequences of risk-adapted therapy for childhood medulloblastoma. *J Clin Oncol, 23*, 5511-5519.

Northcott, P. A., Buchhalter, I., Morrissy, A. S., Hovestadt, V., Weischenfeldt, J., Ehrenberger, T., Gröbner, S., Segura-Wang, M., Zichner, T., Rudneva, V. A., Warnatz, H. J., Sidiropoulos, N., Phillips, A. H., Schumacher, S., Kleinheinz, K., Waszak, S. M., Erkek, S., Jones, D. T. W., Worst, B. C., Kool, M., Zapatka, M., Jäger, N., Chavez, L., Hutter, B., Bieg, M., Paramasivam, N., Heinold, M., Gu, Z., Ishaque, N., Jäger-Schmidt, C., Imbusch, C. D., Jugold, A., Hübschmann, D., Risch, T., Amstislavskiy, V., Gonzalez, F. G. R., Weber, U. D., Wolf, S., Robinson, G. W., Zhou, X., Wu, G., Finkelstein, D., Liu, Y., Cavalli, F. M. G., Luu, B., Ramaswamy, V., Wu, X., Koster, J., Ryzhova, M., Cho, Y. J., Pomeroy, S. L., Herold-Mende, C., Schuhmann, M., Ebinger, M., Liau, L. M., Mora, J., McLendon, R. E., Jabado, N., Kumabe, T., Chuah, E., Ma, Y., Moore, R. A., Mungall, A. J., Mungall, K. L., Thiessen, N., Tse, K., Wong, T., Jones, S. J. M., Witt, O., Milde, T., Von Deimling, A., Capper, D., Korshunov, A., Yaspo, M. L., Kriwacki, R., Gajjar, A., Zhang, J., Beroukhim, R., Fraenkel, E., Korbel, J. O., Brors, B., Schlesner, M., Eils, R., Marra, M. A., Pfister, S. M., Taylor, M.

D., & Lichter, P. (2017). The whole-genome landscape of medulloblastoma subtypes. *Nature, 547*, 311-317.

Northcott, P. A., Hielscher, T., Dubuc, A., Mack, S., Shih, D., Remke, M., Al-Halabi, H., Albrecht, S., Jabado, N., Eberhart, C. G., Grajkowska, W., Weiss, W. A., Clifford, S. C., Bouffet, E., Rutka, J. T., Korshunov, A., Pfister, S., & Taylor, M. D. (2011). Pediatric and adult sonic hedgehog medulloblastomas are clinically and molecularly distinct. *Acta Neuropathol, 122*, 231-240.

Northcott, P. A., Korshunov, A., Pfister, S. M., & Taylor, M. D. (2012). The clinical implications of medulloblastoma subgroups. *Nat Rev Neurol, 8*, 340-351.

Northcott, P. A., Korshunov, A., Witt, H., Hielscher, T., Eberhart, C. G., Mack, S., Bouffet, E., Clifford, S. C., Hawkins, C. E., French, P., Rutka, J. T., Pfister, S., & Taylor, M. D. (2011). Medulloblastoma comprises four distinct molecular variants. *J Clin Oncol, 29*, 1408-1414.

Northcott, P. A., Robinson, G. W., Kratz, C. P., Mabbott, D. J., Pomeroy, S. L., Clifford, S. C., Rutkowski, S., Ellison, D. W., Malkin, D., Taylor, M. D., Gajjar, A., & Pfister, S. M. (2019). Medulloblastoma. *Nat Rev Dis Primers, 5*, 11.

Northcott, P. A., Shih, D. J., Remke, M., Cho, Y. J., Kool, M., Hawkins, C., Eberhart, C. G., Dubuc, A., Guettouche, T., Cardentey, Y., Bouffet, E., Pomeroy, S. L., Marra, M., Malkin, D., Rutka, J. T., Korshunov, A., Pfister, S., & Taylor, M. D. (2012). Rapid, reliable, and reproducible molecular sub-grouping of clinical medulloblastoma samples. *Acta Neuropathol, 123*, 615-626.

Ocasio, J., Babcock, B., Malawsky, D., Weir, S. J., Loo, L., Simon, J. M., Zylka, M. J., Hwang, D., Dismuke, T., Sokolsky, M., Rosen, E. P., Vibhakar, R., Zhang, J., Saulnier, O., Vladoiu, M., El-Hamamy, I., Stein, L. D., Taylor, M. D., Smith, K. S., Northcott, P. A., Colaneri, A., Wilhelmsen, K., & Gershon, T. R. (2019). scRNA-seq in medulloblastoma shows cellular heterogeneity and lineage expansion support resistance to SHH inhibitor therapy. *Nat Commun, 10*, 5829.

Oliver, T. G., Read, T. A., Kessler, J. D., Mehmeti, A., Wells, J. F., Huynh, T. T., Lin, S. M., & Wechsler-Reya, R. J. (2005). Loss of patched and disruption of granule cell development in a pre-neoplastic stage of medulloblastoma. *Development, 132*, 2425-2439.

Park, S. Y., Gönen, M., Kim, H. J., Michor, F., & Polyak, K. (2010). Cellular and genetic diversity in the progression of in situ human breast carcinomas to an invasive phenotype. *J Clin Invest, 120*, 636-644.

Pietsch, T., & Haberler, C. (2016). Update on the integrated histopathological and genetic classification of medulloblastoma - a practical diagnostic guideline. *Clin Neuropathol, 35*, 344-352.

Pizer, B. L., & Clifford, S. C. (2009). The potential impact of tumour biology on improved clinical practice for medulloblastoma: progress towards biologically driven clinical trials. *Br J Neurosurg, 23*, 364-375.

Qian, J., Olbrecht, S., Boeckx, B., Vos, H., Laoui, D., Etlioglu, E., Wauters, E., Pomella, V., Verbandt, S., Busschaert, P., Bassez, A., Franken, A., Bempt, M. V., Xiong, J., Weynand, B., van Herck, Y., Antoranz, A., Bosisio, F. M., Thienpont, B., Floris, G., Vergote, I., Smeets, A., Tejpar, S., & Lambrechts, D. (2020). A pan-cancer blueprint of the heterogeneous tumor microenvironment revealed by single-cell profiling. *Cell Res*.

Raj, A., & van Oudenaarden, A. (2008). Nature, nurture, or chance: stochastic gene expression and its consequences. *Cell, 135*, 216-226.

Ramaswamy, V., Remke, M., Bouffet, E., Bailey, S., Clifford, S. C., Doz, F., Kool, M., Dufour, C., Vassal, G., Milde, T., Witt, O., von Hoff, K., Pietsch, T., Northcott, P. A., Gajjar, A., Robinson, G. W., Padovani, L., Andre, N., Massimino, M., Pizer, B., Packer, R., Rutkowski,

S., Pfister, S. M., Taylor, M. D., & Pomeroy, S. L. (2016). Risk stratification of childhood medulloblastoma in the molecular era: the current consensus. *Acta Neuropathol, 131*, 821-831.

Rausch, T., Jones, D. T., Zapatka, M., Stütz, A. M., Zichner, T., Weischenfeldt, J., Jäger, N., Remke, M., Shih, D., Northcott, P. A., Pfaff, E., Tica, J., Wang, Q., Massimi, L., Witt, H., Bender, S., Pleier, S., Cin, H., Hawkins, C., Beck, C., von Deimling, A., Hans, V., Brors, B., Eils, R., Scheurlen, W., Blake, J., Benes, V., Kulozik, A. E., Witt, O., Martin, D., Zhang, C., Porat, R., Merino, D. M., Wasserman, J., Jabado, N., Fontebasso, A., Bullinger, L., Rücker, F. G., Döhner, K., Döhner, H., Koster, J., Molenaar, J. J., Versteeg, R., Kool, M., Tabori, U., Malkin, D., Korshunov, A., Taylor, M. D., Lichter, P., Pfister, S. M., & Korbel, J. O. (2012). Genome sequencing of pediatric medulloblastoma links catastrophic DNA rearrangements with TP53 mutations. *Cell, 148*, 59-71.

Riley, R. S., June, C. H., Langer, R., & Mitchell, M. J. (2019). Delivery technologies for cancer immunotherapy. *Nat Rev Drug Discov, 18*, 175-196.

Robinson, G. W., Orr, B. A., Wu, G., Gururangan, S., Lin, T., Qaddoumi, I., Packer, R. J., Goldman, S., Prados, M. D., Desjardins, A., Chintagumpala, M., Takebe, N., Kaste, S. C., Rusch, M., Allen, S. J., Onar-Thomas, A., Stewart, C. F., Fouladi, M., Boyett, J. M., Gilbertson, R. J., Curran, T., Ellison, D. W., & Gajjar, A. (2015). Vismodegib Exerts Targeted Efficacy Against Recurrent Sonic Hedgehog-Subgroup Medulloblastoma: Results From Phase II Pediatric Brain Tumor Consortium Studies PBTC-025B and PBTC-032. *J Clin Oncol, 33*, 2646-2654.

Robinson, G. W., Rudneva, V. A., Buchhalter, I., Billups, C. A., Waszak, S. M., Smith, K. S., Bowers, D. C., Bendel, A., Fisher, P. G., Partap, S., Crawford, J. R., Hassall, T., Indelicato, D. J., Boop, F., Klimo, P., Sabin, N. D., Patay, Z., Merchant, T. E., Stewart, C. F., Orr, B. A., Korbel, J. O., Jones, D. T. W., Sharma, T., Lichter, P., Kool, M., Korshunov, A., Pfister, S. M., Gilbertson, R. J., Sanders, R. P., Onar-Thomas, A., Ellison, D. W., Gajjar, A., & Northcott, P. A. (2018). Risk-adapted therapy for young children with medulloblastoma (SJYC07): therapeutic and molecular outcomes from a multicentre, phase 2 trial. *Lancet Oncol, 19*, 768-784.

Rodriguez-Mateos, P., Azevedo, N. F., Almeida, C., & Pamme, N. (2020). FISH and chips: a review of microfluidic platforms for FISH analysis. *Med Microbiol Immunol, 209*, 373-391.

Rorke, L. B. (1983). The cerebellar medulloblastoma and its relationship to primitive neuroectodermal tumors. *J Neuropathol Exp Neurol, 42*, 1-15.

Rutkowski, S., von Hoff, K., Emser, A., Zwiener, I., Pietsch, T., Figarella-Branger, D., Giangaspero, F., Ellison, D. W., Garre, M. L., Biassoni, V., Grundy, R. G., Finlay, J. L., Dhall, G., Raquin, M. A., & Grill, J. (2010). Survival and prognostic factors of early childhood medulloblastoma: an international meta-analysis. *J Clin Oncol, 28*, 4961-4968.

Ryan, S. L., Schwalbe, E. C., Cole, M., Lu, Y., Lusher, M. E., Megahed, H., O'Toole, K., Nicholson, S. L., Bognar, L., Garami, M., Hauser, P., Korshunov, A., Pfister, S. M., Williamson, D., Taylor, R. E., Ellison, D. W., Bailey, S., & Clifford, S. C. (2012). MYC family amplification and clinical risk-factors interact to predict an extremely poor prognosis in childhood medulloblastoma. *Acta Neuropathol, 123*, 501-513.

Schwalbe, E. C., Lindsey, J. C., Nakjang, S., Crosier, S., Smith, A. J., Hicks, D., Rafiee, G., Hill, R. M., Iliasova, A., Stone, T., Pizer, B., Michalski, A., Joshi, A., Wharton, S. B., Jacques, T. S., Bailey, S., Williamson, D., & Clifford, S. C. (2017). Novel molecular subgroups for clinical classification and outcome prediction in childhood medulloblastoma: a cohort study. *Lancet Oncol, 18*, 958-971.

Schwalbe, E. C., Lindsey, J. C., Straughton, D., Hogg, T. L., Cole, M., Megahed, H., Ryan, S. L., Lusher, M. E., Taylor, M. D., Gilbertson, R. J., Ellison, D. W., Bailey, S., & Clifford, S. C. (2011). Rapid diagnosis of medulloblastoma molecular subgroups. *Clin Cancer Res, 17*, 1883-1894.

Sharma, T., Schwalbe, E. C., Williamson, D., Sill, M., Hovestadt, V., Mynarek, M., Rutkowski, S., Robinson, G. W., Gajjar, A., Cavalli, F., Ramaswamy, V., Taylor, M. D., Lindsey, J. C., Hill, R. M., Jäger, N., Korshunov, A., Hicks, D., Bailey, S., Kool, M., Chavez, L., Northcott, P. A., Pfister, S. M., & Clifford, S. C. (2019). Second-generation molecular subgrouping of medulloblastoma: an international meta-analysis of Group 3 and Group 4 subtypes. *Acta Neuropathol, 138*, 309-326.

Silva, R., Marie, S. K., Uno, M., Matushita, H., Wakamatsu, A., Rosemberg, S., & Oba-Shinjo, S. M. (2013). CTNNB1, AXIN1 and APC expression analysis of different medulloblastoma variants. *Clinics (Sao Paulo), 68*, 167-172.

Taylor, A. M., Shih, J., Ha, G., Gao, G. F., Zhang, X., Berger, A. C., Schumacher, S. E., Wang, C., Hu, H., Liu, J., Lazar, A. J., Cherniack, A. D., Beroukhim, R., & Meyerson, M. (2018). Genomic and Functional Approaches to Understanding Cancer Aneuploidy. *Cancer Cell, 33*, 676-689.e673.

Taylor, M. D., Northcott, P. A., Korshunov, A., Remke, M., Cho, Y. J., Clifford, S. C., Eberhart, C. G., Parsons, D. W., Rutkowski, S., Gajjar, A., Ellison, D. W., Lichter, P., Gilbertson, R. J., Pomeroy, S. L., Kool, M., & Pfister, S. M. (2012). Molecular subgroups of medulloblastoma: the current consensus. *Acta Neuropathol, 123*, 465-472.

Terry, R. L., Meyran, D., Ziegler, D. S., Haber, M., Ekert, P. G., Trapani, J. A., & Neeson, P. J. (2020). Immune profiling of pediatric solid tumors. *J Clin Invest, 130*, 3391-3402.

Thompson, E. M., Bramall, A., Herndon, J. E., 2nd, Taylor, M. D., & Ramaswamy, V. (2018). The clinical importance of medulloblastoma extent of resection: a systematic review. *J Neurooncol, 139*, 523-539.

Thompson, E. M., Hielscher, T., Bouffet, E., Remke, M., Luu, B., Gururangan, S., McLendon, R. E., Bigner, D. D., Lipp, E. S., Perreault, S., Cho, Y. J., Grant, G., Kim, S. K., Lee, J. Y., Rao, A. A. N., Giannini, C., Li, K. K. W., Ng, H. K., Yao, Y., Kumabe, T., Tominaga, T., Grajkowska, W. A., Perek-Polnik, M., Low, D. C. Y., Seow, W. T., Chang, K. T. E., Mora, J., Pollack, I. F., Hamilton, R. L., Leary, S., Moore, A. S., Ingram, W. J., Hallahan, A. R., Jouvet, A., Fevre-Montange, M., Vasiljevic, A., Faure-Conter, C., Shofuda, T., Kagawa, N., Hashimoto, N., Jabado, N., Weil, A. G., Gayden, T., Wataya, T., Shalaby, T., Grotzer, M., Zitterbart, K., Sterba, J., Krskova, L., Hortobagyi, T., Klekner, A., Laszlo, B., Pocza, T., Hauser, P., Schuller, U., Jung, S., Jang, W. Y., French, P. J., Kros, J. M., van Veelen, M. C., Massimi, L., Leonard, J. R., Rubin, J. B., Vibhakar, R., Chambless, L. B., Cooper, M. K., Thompson, R. C., Faria, C. C., Carvalho, A., Nunes, S., Pimentel, J., Fan, X., Muraszko, K. M., Lopez-Aguilar, E., Lyden, D., Garzia, L., Shih, D. J. H., Kijima, N., Schneider, C., Adamski, J., Northcott, P. A., Kool, M., Jones, D. T. W., Chan, J. A., Nikolic, A., Garre, M. L., Van Meir, E. G., Osuka, S., Olson, J. J., Jahangiri, A., Castro, B. A., Gupta, N., Weiss, W. A., Moxon-Emre, I., Mabbott, D. J., Lassaletta, A., Hawkins, C. E., Tabori, U., Drake, J., Kulkarni, A., Dirks, P., Rutka, J. T., Korshunov, A., Pfister, S. M., Packer, R. J., Ramaswamy, V., & Taylor, M. D. (2016). Prognostic value of medulloblastoma extent of resection after accounting for molecular subgroup: a retrospective integrated clinical and molecular analysis. *Lancet Oncol, 17*, 484-495.

Thompson, M. C., Fuller, C., Hogg, T. L., Dalton, J., Finkelstein, D., Lau, C. C., Chintagumpala, M., Adesina, A., Ashley, D. M., Kellie, S. J., Taylor, M. D., Curran, T., Gajjar, A., & Gilbertson, R. J. (2006). Genomics identifies medulloblastoma subgroups that are enriched for specific genetic alterations. *J Clin Oncol, 24*, 1924-1931.

Thrane, K., Eriksson, H., Maaskola, J., Hansson, J., & Lundeberg, J. (2018). Spatially Resolved Transcriptomics Enables Dissection of Genetic Heterogeneity in Stage III Cutaneous Malignant Melanoma. *Cancer Res, 78*, 5970-5979.

Turajlic, S., Sottoriva, A., Graham, T., & Swanton, C. (2019). Resolving genetic heterogeneity in cancer. *Nat Rev Genet, 20*, 404-416.

Vladoiu, M. C., El-Hamamy, I., Donovan, L. K., Farooq, H., Holgado, B. L., Sundaravadanam, Y., Ramaswamy, V., Hendrikse, L. D., Kumar, S., Mack, S. C., Lee, J. J. Y., Fong, V., Juraschka, K., Przelicki, D., Michealraj, A., Skowron, P., Luu, B., Suzuki, H., Morrissy, A. S., Cavalli, F. M. G., Garzia, L., Daniels, C., Wu, X., Qazi, M. A., Singh, S. K., Chan, J. A., Marra, M. A., Malkin, D., Dirks, P., Heisler, L., Pugh, T., Ng, K., Notta, F., Thompson, E. M., Kleinman, C. L., Joyner, A. L., Jabado, N., Stein, L., & Taylor, M. D. (2019). Childhood cerebellar tumours mirror conserved fetal transcriptional programs. *Nature, 572*, 67-73.

Wang, J., Cazzato, E., Ladewig, E., Frattini, V., Rosenbloom, D. I., Zairis, S., Abate, F., Liu, Z., Elliott, O., Shin, Y. J., Lee, J. K., Lee, I. H., Park, W. Y., Eoli, M., Blumberg, A. J., Lasorella, A., Nam, D. H., Finocchiaro, G., Iavarone, A., & Rabadan, R. (2016). Clonal evolution of glioblastoma under therapy. *Nat Genet, 48*, 768-776.

Wang, X., Ramaswamy, V., Remke, M., Mack, S. C., Dubuc, A. M., Northcott, P. A., & Taylor, M. D. (2013). Intertumoral and intratumoral heterogeneity as a barrier for effective treatment of medulloblastoma. *Neurosurgery, 59 Suppl 1*, 57-63.

Wang, Y., Waters, J., Leung, M. L., Unruh, A., Roh, W., Shi, X., Chen, K., Scheet, P., Vattathil, S., Liang, H., Multani, A., Zhang, H., Zhao, R., Michor, F., Meric-Bernstam, F., & Navin, N. E. (2014). Clonal evolution in breast cancer revealed by single nucleus genome sequencing. *Nature, 512*, 155-160.

Waszak, S. M., Robinson, G. W., Gudenas, B. L., Smith, K. S., Forget, A., Kojic, M., Garcia-Lopez, J., Hadley, J., Hamilton, K. V., Indersie, E., Buchhalter, I., Kerssemakers, J., Jager, N., Sharma, T., Rausch, T., Kool, M., Sturm, D., Jones, D. T. W., Vasilyeva, A., Tatevossian, R. G., Neale, G., Lombard, B., Loew, D., Nakitandwe, J., Rusch, M., Bowers, D. C., Bendel, A., Partap, S., Chintagumpala, M., Crawford, J., Gottardo, N. G., Smith, A., Dufour, C., Rutkowski, S., Eggen, T., Wesenberg, F., Kjaerheim, K., Feychting, M., Lannering, B., Schuz, J., Johansen, C., Andersen, T. V., Roosli, M., Kuehni, C. E., Grotzer, M., Remke, M., Puget, S., Pajtler, K. W., Milde, T., Witt, O., Ryzhova, M., Korshunov, A., Orr, B. A., Ellison, D. W., Brugieres, L., Lichter, P., Nichols, K. E., Gajjar, A., Wainwright, B. J., Ayrault, O., Korbel, J. O., Northcott, P. A., & Pfister, S. M. (2020). Germline Elongator mutations in Sonic Hedgehog medulloblastoma. *Nature, 580*, 396-401.

Xu, X., Hou, Y., Yin, X., Bao, L., Tang, A., Song, L., Li, F., Tsang, S., Wu, K., Wu, H., He, W., Zeng, L., Xing, M., Wu, R., Jiang, H., Liu, X., Cao, D., Guo, G., Hu, X., Gui, Y., Li, Z., Xie, W., Sun, X., Shi, M., Cai, Z., Wang, B., Zhong, M., Li, J., Lu, Z., Gu, N., Zhang, X., Goodman, L., Bolund, L., Wang, J., Yang, H., Kristiansen, K., Dean, M., Li, Y., & Wang, J. (2012). Single-cell exome sequencing reveals single-nucleotide mutation characteristics of a kidney tumor. *Cell, 148*, 886-895.

Yang, Z. J., Ellis, T., Markant, S. L., Read, T. A., Kessler, J. D., Bourboulas, M., Schuller, U., Machold, R., Fishell, G., Rowitch, D. H., Wainwright, B. J., & Wechsler-Reya, R. J. (2008). Medulloblastoma can be initiated by deletion of Patched in lineage-restricted progenitors or stem cells. *Cancer Cell, 14*, 135-145.

Yates, L. R., & Campbell, P. J. (2012). Evolution of the cancer genome. *Nat Rev Genet, 13*, 795-806.

Ye, C. J., Stevens, J. B., Liu, G., Bremer, S. W., Jaiswal, A. S., Ye, K. J., Lin, M. F., Lawrenson, L., Lancaster, W. D., Kurkinen, M., Liao, J. D., Gairola, C. G., Shekhar, M. P., Narayan, S., Miller, F. R., & Heng, H. H. (2009). Genome based cell population heterogeneity

promotes tumorigenicity: the evolutionary mechanism of cancer. *J Cell Physiol, 219*, 288-300.

Zahn, H., Steif, A., Laks, E., Eirew, P., VanInsberghe, M., Shah, S. P., Aparicio, S., & Hansen, C. L. (2017). Scalable whole-genome single-cell library preparation without preamplification. *Nat Methods, 14*, 167-173.

Zeltzer, P. M., Boyett, J. M., Finlay, J. L., Albright, A. L., Rorke, L. B., Milstein, J. M., Allen, J. C., Stevens, K. R., Stanley, P., Li, H., Wisoff, J. H., Geyer, J. R., McGuire-Cullen, P., Stehbens, J. A., Shurin, S. B., & Packer, R. J. (1999). Metastasis stage, adjuvant treatment, and residual tumor are prognostic factors for medulloblastoma in children: conclusions from the Children's Cancer Group 921 randomized phase III study. *J Clin Oncol, 17*, 832-845.

Zhang, L., He, X., Liu, X., Zhang, F., Huang, L. F., Potter, A. S., Xu, L., Zhou, W., Zheng, T., Luo, Z., Berry, K. P., Pribnow, A., Smith, S. M., Fuller, C., Jones, B. V., Fouladi, M., Drissi, R., Yang, Z. J., Gustafson, W. C., Remke, M., Pomeroy, S. L., Girard, E. J., Olson, J. M., Morrissy, A. S., Vladoiu, M. C., Zhang, J., Tian, W., Xin, M., Taylor, M. D., Potter, S. S., Roussel, M. F., Weiss, W. A., & Lu, Q. R. (2019). Single-Cell Transcriptomics in Medulloblastoma Reveals Tumor-Initiating Progenitors and Oncogenic Cascades during Tumorigenesis and Relapse. *Cancer Cell, 36*, 302-318.e307.

Zhukova, N., Ramaswamy, V., Remke, M., Pfaff, E., Shih, D. J., Martin, D. C., Castelo-Branco, P., Baskin, B., Ray, P. N., Bouffet, E., von Bueren, A. O., Jones, D. T., Northcott, P. A., Kool, M., Sturm, D., Pugh, T. J., Pomeroy, S. L., Cho, Y. J., Pietsch, T., Gessi, M., Rutkowski, S., Bognar, L., Klekner, A., Cho, B. K., Kim, S. K., Wang, K. C., Eberhart, C. G., Fevre-Montange, M., Fouladi, M., French, P. J., Kros, M., Grajkowska, W. A., Gupta, N., Weiss, W. A., Hauser, P., Jabado, N., Jouvet, A., Jung, S., Kumabe, T., Lach, B., Leonard, J. R., Rubin, J. B., Liau, L. M., Massimi, L., Pollack, I. F., Shin Ra, Y., Van Meir, E. G., Zitterbart, K., Schuller, U., Hill, R. M., Lindsey, J. C., Schwalbe, E. C., Bailey, S., Ellison, D. W., Hawkins, C., Malkin, D., Clifford, S. C., Korshunov, A., Pfister, S., Taylor, M. D., & Tabori, U. (2013). Subgroup-specific prognostic implications of TP53 mutation in medulloblastoma. *J Clin Oncol, 31*, 2927-2935.