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Medulloblastomics revisited: biological and clinical insights from thousands of patients

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Abstract

Medulloblastoma, a malignant childhood brain tumour, has been the recent focus of intensive molecular profiling efforts, profoundly advancing our understanding of biologically and clinically heterogeneous disease subgroups. Genomic, epigenomic, transcriptomic, and proteomic landscapes have now been mapped for an unprecedented number of bulk medulloblastoma patient samples, and more recently single medulloblastoma cells. These efforts have provided pivotal new insights into the diverse molecular mechanisms presumed to drive tumour initiation, maintenance, and recurrence across individual subgroups and subtypes. Translational opportunities stemming from this knowledge are continuing to evolve, providing a framework for improved diagnostic and therapeutic intervention. In this Review, we summarize recent advances illuminated through continued molecular characterization of medulloblastoma and contextualize this progress towards the deployment of more effective, molecularly informed treatments for affected children.

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Competing interests

None.

Introduction

Medulloblastoma (MB), a pediatric cerebellar tumour, is one of the most common malignant central nervous system (CNS) tumours in children (~500 new diagnoses/year in the United States) and a leading cause of cancer-related death in this age group[1]. During the past two decades, large-scale genomic efforts have helped disentangle the molecular basis of MB, especially biologically and clinically relevant intertumoural heterogeneity. Consensus molecular subgroups[2], WNT, SHH, Group 3, and Group 4, each characterized by distinct ‘omic and clinical features, are now widely recognized. As a direct consequence, mechanistic, developmental, and preclinical studies are currently undertaken in a manner that is cognizant of molecular subgroup status. Moreover, clinical protocols have adopted molecular subgrouping strategies into routine diagnosis, treatment stratification, and patient selection for molecularly targeted therapies.

In 2012, following the formal recognition of consensus molecular subgroups, the initial wave of next-generation sequencing (NGS) studies conducted on primary MB samples were reported[3–7]. Since then, additional molecular characterization has ensued on increasingly large cohorts, resulting in the generation of a wealth of multidimensional ‘omics data. As a result, molecular classification of MB has evolved beyond the four consensus subgroups and new methods for robust and accurate assignment of patients into relevant subtypes have become mainstream. More recently, spatial and single-cell genomic approaches have been applied, delving into intratumoural heterogeneity, cellular composition, and developmental origins at single-cell resolution. We recently reviewed the epidemiologic, biologic, and therapeutic characteristics of MB in a report that was intended to be all encompassing of the human disease[1]. In this Review, we emphasize what has been learned from the ‘omic analysis of MB patients since the first NGS studies[3], highlighting insights into the molecular and biological mechanisms underlying MB heterogeneity and translational implications emerging from these efforts.

Molecular Classification

WHO Consensus subgroups & subtypes within subgroups

First gene expression array studies confirmed that MB was molecularly distinct from other embryonal brain tumours, such as primitive neuroectodermal tumour (PNET) and atypical teratoid rhabdoid tumour (AT/RT)[8]. In this seminal report, gene expression comparison of classic versus desmoplastic histology MB identified specific up-regulation of genes involved in hedgehog signaling in desmoplastic tumours, including *PTCH1*, *GLI1*, and *MYCN*[8]. A parallel report comparing expression profiles of a series of genetically engineered mouse (GEM) models to normal cerebellar controls identified similar activation of hedgehog pathway-associated gene sets in MB GEM models[9]. Together, these early reports of gene expression signatures defining MBs in humans and mice would pave the way for the molecular era that would follow. Multiple independent gene expression array profiling studies conducted on patient cohorts described distinct molecular subgroups within MB that differ in their demographics, genetic alterations, and clinical outcomes[10–13], culminating in the definition of consensus subgroups[2]. The WNT and SHH subgroups, which represent approximately 10% and 30% of all MB patients, respectively, are associated with activation

of the WNT (Wingless) and SHH (Sonic hedgehog) signaling pathways. WNT and SHH subgroups are discrete across studies and technologies, providing a basis for their incorporation into the 2016 update of the WHO Classification of Central Nervous System Tumors[14]. Further subdivision within the SHH subgroup according to *TP53* mutation status (SHH-*TP53* wild type and SHH-*TP53* mutant) is also recognized by the WHO. Group 3 and Group 4 subgroups, which represent the remaining 25% and 35% of all MB patients, respectively, exhibit some molecular and biological similarities (detailed below). Consequently, these subgroups are formally recognized by the WHO as non-WNT/non-SHH-MB, listing Group 3 and Group 4 designations as provisional entities.

Differential expression analysis between MB subgroups has led to the identification of select biomarkers that enable molecular classification based on immunohistochemistry[12,15,16], NanoString[17,18], and other panel-based gene expression assays[19]. While these assays are widely accessible in clinical practice, DNA methylation arrays, which can measure hundreds of thousands of methylation sites across the genome, have emerged as the platform of choice for MB classification. Owing to the relative stability of DNA, methylation arrays and derivative targeted DNA methylation-based assays, allow for routine analysis of clinical samples in a diagnostic setting as well as profiling of archival tumour material, with limited technical variation between institutions[20–24].

In 2017, three independent studies[25–27] used DNA methylation analysis to investigate additional substructure within subgroups, leading to a varying definition of molecular subtypes in each study (Figure 1; detailed below).

WNT medulloblastoma.

WNT-MB primarily occurs in children after 4 years of age to early adulthood (median age ~11 years) and exhibits a balanced male:female ratio (Figure 2). WNT-MB tumours are usually of classic histology and infrequently metastatic at diagnosis. Outcomes for WNT-MB patients are favorable, experiencing 5-year survival rates of 95% or better. Somatic mutations in *CTNNB1* are the hallmark genetic event defining this subgroup (~85% of patients)[13,28], with most remaining patients typified by pathogenic *APC* variants in the germline[29]. Tumour genomes are mostly devoid of somatic copy-number alterations (SCNAs), except for loss of chromosome 6 (i.e. monosomy 6) in most patients.

WNT-MB has been described to be largely homogenous between patients in regard to genome-wide expression and methylation profiles. However, two molecular subtypes, WNT α and WNT β, that differ in age at diagnosis (median age of 10 vs. 20 years, respectively) and frequency of monosomy 6 have been suggested[25]. Survival outcomes of adults with WNT-MB (i.e. WNT β[25]) have been inconsistent in the literature, with some reports describing outcomes to be comparably favorable to those of pediatric WNT-MB patients[25,30], and others reporting reduced overall survival[31].

SHH medulloblastoma.

SHH-MB displays an intriguing bimodal age distribution, representing the most common molecular subgroup in infants (<3 years of age) and adults (>17 years of age), with fewer cases diagnosed during childhood and adolescence (Figure 2). Demographically,

SHH-MB is more common in males than females (approximately 2:1, male:female). Classic and desmoplastic/nodular (including medulloblastoma with extensive nodularity, MBEN) histology occur at similar frequencies (each accounting for ~40% of patients), with large-cell/anaplastic (LCA) histology making up the remainder. Mutations and focal SCNAs targeting genes in the SHH signaling pathway represent the most common genetic events, including inactivating germline or somatic mutations and deletions in *PTCH1* and *SUFU*, activating mutations in *SMO*, and amplifications of *GLI2*[32]. Frequent chromosomal alterations include loss of chromosomes 9q, 10q, 14q, and 17p, and gains of chromosomes 2 and 9p.

Age-associated molecular differences discriminating infant and adult SHH-MB have been observed by both gene expression and DNA methylation array profiling[33,27,32]. Compared to pediatric counterparts, adult SHH-MBs are characterized by a higher overall mutational burden, a higher prevalence of SHH-pathway associated mutations (including a higher incidence of *PTCH1* and *SMO* alterations*)*, a more expansive list of chromatin modifier mutations (i.e. *BRPF1, CREBBP*), and virtually all harbor *TERT* promoter mutations[32,34–36]. Intriguingly, transcriptional comparison of available SHH-MB mouse models to patient tumours suggested that, despite being primarily driven by inactivation of *Ptch1* or activation of *Smo* – genetic events that occur in both pediatric and adult tumours, current mouse models are more molecularly similar to adult SHH-MB[37].

More recently, four molecular subtypes of SHH-MB have been reported[25] (Figure 1 & 2): SHH-β and SHH-γ correspond to infant subtypes (median age of 1.9 and 1.3 years, respectively), whereas SHH-α and SHH-δ respectively correspond to childhood/adolescent and adult subtypes (median age of 8 and 26 years, respectively). Subtype SHH-α is enriched for patients harboring *TP53* mutations (~1/3 of SHH-α patients) and associated with an inferior outcome compared to SHH-δ patients. Infant subtype SHH-β showed lower 5-year survival than SHH-γ. Similar observations were made in an independent study reporting on a clinical trial in infants and young children (under 6 years of age) which identified two molecular subtypes, iSHH-I (equivalent to SHH-β) and iSHH-II (equivalent to SHH-γ and SHH-α)[38] (Figure 1 & 2).

Group 3/4 medulloblastoma.

Group 3-MB occurs during infancy and childhood and is rarely seen in patients older than 18 years of age, whereas Group 4-MB occurs across all age groups (Figure 2). Male:female ratios are 2:1 or higher for both subgroups. LCA histology is more prevalent in Group 3 than Group 4, and 30–40% of patients are metastatic at diagnosis in both subgroups. *MYC* amplification is a common genetic feature of Group 3-MB and is associated with a particularly poor clinical outcome[10,12]. *MYCN* and *CDK6* amplifications are notable genetic alterations seen in Group 4-MB[6]. Isochromosome 17q is a hallmark cytogenetic event in both subgroups, found in >50% of patients from either subgroup.

The definition and substructure of Group 3/4-MB has been a topic of debate since their initial discovery[10–13]. Indeed, early nomenclature did not always define Group 3 and Group 4 into distinct subgroups and in some cases described them as a single ‘mixed’ subgroup of patients designated as ‘non-WNT/non-SHH’ MB[16]. However, the recognition of Group

3 and Group 4 MB has continued to evolve in recent years, with the majority of the neuro-oncology community, and the WHO[14], acknowledging their definition as mostly discrete molecular entities and supporting the utility of their distinction, as supported in the literature (see[1,39–41] for recent reviews).

More recently, three independent studies identified a varying number of subtypes within Group 3 and Group 4 (Figure 1). Schwalbe and colleagues identified four molecular subtypes in Group 3- and Group 4-MB that split each subgroup into high- and low-risk subtypes[27]. Cavalli and colleagues identified three molecular subtypes in each subgroup: Group 3α, β, and γ, and Group 4α, β, and γ[25]. In a combined analysis of Group 3 and Group 4 cases, Northcott and colleagues identified eight molecular subtypes, designated I to VIII[26]. In order to harmonize subtype definitions, a joint analysis of 1,501 Group 3/4 MBs from all three studies was recently undertaken[42]. This analysis showed that separation into eight subtypes best unified the substructure observed in each of the aforementioned studies (Figure 1).

Subtype I represents the least common subtype and comprises a mix of Group 3 and Group 4 tumours (Figure 2). Subtype I tumour genomes are generally balanced and enriched for amplification of the *OTX2* oncogene and activation of *GFI1/GFI1B* (described in detail below). Subtypes II, III and IV are *bona fide* Group 3 subtypes. Subtypes II and III are characterized by amplification of the *MYC* oncogene and are associated with poor outcomes. In comparison to other subtypes, Subtype IV is enriched for younger patients (median age of 3 years) and is associated with a favorable outcome; low progression free survival observed in infant Subtype IV patients suggests that survival rates are dependent on treatment with craniospinal axis radiation[38]. Subtypes V, VI and VII consist mostly of Group 4 MBs, but also include some Group 3 tumours. Subtype V genomes are characterized by amplification of both *MYC* and *MYCN* and are associated with modest outcomes. Subtype VIII is the most common and only pure Group 4 subtype, mostly occurring in older children (median age of 10 years). Subtype VIII tumours display a balanced genome, except for presence of isochromosome 17 (i17q) in most cases. Subtype VIII is associated with favorable 5-year survival; however, many patients are affected by late relapse and death, a feature that is unique to this subtype[42].

Genome

Gene mutations.

MB NGS studies primarily detailed somatic non-synonymous mutations affecting protein-coding genes in relatively modest patient cohorts[4,5,7,43]. New recurrently mutated genes emerged from these analyses, including *DDX3X*, *BCOR*, *CTDNEP1*, and *TBR1*, among others. In addition, chromatin-modifying genes such as *KMT2D* (*MLL2*), *KMT2C* (*MLL3*), *SMARCA4*, and *KDM6A*, previously identified by large-scale exome-resequencing[44], were also confirmed and their mutational frequencies and distribution contextualized by subgroup. Despite these advances, initial standalone studies were underpowered to adequately detail the broader scope of low frequency driver gene alterations contributing to MB, especially in Groups 3 and 4 which were heterogeneous and devoid of highly recurrent gene-centric mutations[3].

Recently, an international collaborative effort aimed at comprehensive characterization of the MB genomic landscape summarized putative driver gene alterations across a series of 491 primary MB samples[26]. As expected, WNT- and SHH-MB subgroups were largely characterized by mutations and SCNAs affecting known genes (Figure 3). Of interest, functional annotation of recurrently altered genes identified somatic deregulation of SWI/SNF family chromatin remodeling genes in one third of WNT-MBs (namely *SMARCA4*, *ARID1A*, and *ARID2*) and recurrent targeting of histone acetyltransferase genes in nearly 20% of SHH-MBs (namely *CREBBP*, *KANSL1*, *BRPF1*, and others). Detailed mechanistic studies will be required to determine how somatic targeting of these chromatin-associated complexes potentiates MB pathogenesis in the affected subgroups.

In Group 3/4-MB, the multidimensional molecular analysis performed on this broader set of patients (n=324 total) confirmed that recurrent gene-level mutations remained relatively rare (Figure 3). *SMARCA4* mutations were seen in 9% of Group 3 and only 2% of Group 4 patients. *KDM6A* (7%), *ZMYM3* (6%), and *KMT2C* (6%) represented the most commonly mutated genes in Group 4. Previously unknown somatic in-frame insertions affecting *KBTBD4* were evenly distributed between Group 3- and Group 4-MB (6% of patients from either subgroup). Although poorly characterized to date, KBTBD4 is a member of the BTB-BACK-Kelch protein family and predicted to recruit protein substrates to cullin-RING ligases for targeted ubiquitination and protein degradation[45]. MB-associated *KBTBD4* insertions were confined to the Kelch domain and deemed unlikely to disrupt the overall domain structure but instead converged on the known substrate-binding interface described for other BTB-BACK-Kelch protein family members. Recently, identical somatic in-frame insertions to those seen in MB have been reported in pineal parenchymal tumours of intermediate differentiation (PPTID)[46], suggesting that common oncogenic mechanism(s) may be shared between affected MB and PPTID patients.

Structural alterations and enhancer hijacking.

Group 3/4-MB genomes are characterized by a preponderance of SCNAs and structural variants (SVs)[6], suggesting that these alterations play an integral role in disease pathogenesis. Analyzing the genomes of 137 Group 3/4-MB samples identified a series of atypical SVs (i.e. deletions, duplications, inversions, and more complex genomic alterations) mapping to chromosome 9q34 that were specific to these subgroups[47]. Integration with sample-matched gene expression data uncovered pronounced, SV-associated up-regulation of GFI1B in affected samples. GFI1B is a transcriptional repressor that is primarily known for its role in T-cell and B-cell development, as well as in hematopoietic malignancies where it functions as an oncogene[48]. The related family member GFI1, was also determined to be aberrantly expressed in a mutually exclusive set of Group 3/4-MBs harboring SV breakpoints proximal to the *GFI1* locus. Integration with histone chromatin immunoprecipitation sequencing (ChIP-seq) data for H3K27ac marking active enhancers, in addition to other supporting ‘omics data, suggested that activation of GFI1B and GFI1 expression in MB was accomplished via SV-dependent misappropriation of distal, highly active enhancers/super-enhancers to their normally repressed gene promoters. Similar to classical translocations leading to over-expression of established oncogenes, such as IgG-MYC in Burkitt lymphoma[49,50], this mechanism of SV-dependent gene activation was

designated ‘enhancer hijacking’ and has since been explored and documented in numerous follow-up studies in other cancer types[51–55]. Using an orthotopic transplantation approach, GFI1B and GFI1 were validated as novel MB oncogenes capable of cooperating with MYC to promote highly aggressive Group 3-like MB in mice[47]. Overall, enhancer hijacking-associated GFI1 and GFI1B activation is estimated to account for ~12–15% of Group 3/4-MB patients, with clear enrichment of these events in Subtype I and to a lesser extent Subtype II[26].

Motivated by the discovery of enhancer hijacking above, a novel computational pipeline termed CESAM (*Cis*-expression structural alteration mapping)[54] was developed to systematically identify additional enhancer hijacking events through integration of SV breakpoints and gene expression data. Applying CESAM to sample-matched genomic datasets derived from 164 MB patients discovered PRDM6 as a novel target of enhancer hijacking in 17% of Group 4 patients[26]. *PRDM6* maps to chromosome 5q23, approximately 600kb downstream of *SNCAIP*, a locus known to be targeted by highly recurrent, stereotypical tandem duplications exclusively in Group 4-MB[6]. Through multi-omic data integration for a series of Group 4-MBs, a putative model of enhancer hijacking mediated activation of PRDM6 was proposed. PRDM6 is described as a transcriptional repressor, mediating gene silencing through intrinsic H4K20 methyltransferase activity in concert with known chromatin-associated repressive complexes[56,57]. To date, PRDM6 represents the most frequent somatically altered gene in Group 4-MB, and together with mutations targeting other chromatin-modifying genes, further implicates deregulation of physiological transcriptional control as an essential mechanism underlying Group 4-MB pathogenesis.

Transcriptome & Epigenome

Compared to childhood leukemias[58] and other pediatric brain cancers (i.e. supratentorial ependymoma[59], pilocytic astrocytoma[60]), recurrent gene fusions are rare in MB. Early transcriptome sequencing discovered recurrent *PVT1* gene fusions in Group 3-MB that were linked to chromothripsis and *MYC* amplification on chromosome 8q24[6]. Additional *PVT1*-associated fusion events have since been reported in Group 3, including *PVT1-NDRG1*, *PVT1-LINC00964*, *PVT1-ZCH3*, and others[26]. *PVT1* encodes a long intergenic noncoding RNA (lincRNA) harboring a cluster of six annotated microRNAs (namely, miR-1204, miR-1205, miR-1206, miR-1207–5p, miR-1207–3p, and miR-1208). Several reports link *PVT1* over-expression and activity with pro-tumourigenic phenotypes[61–67]. In contrast, a recent study suggested that the *PVT1* gene promoter is a tumour suppressor DNA element that inhibits *MYC* expression through enhancer-promoter competition *in cis*[68]. Future mechanistic and phenotypic studies in relevant model systems will be necessary to decipher the role of *PVT1* fusions, among others, seen in MB.

Transcriptome analysis of MB patient samples has also led to the identification of alternate promoters and transcriptional start sites (TSS), indicating that transcriptional regulation of specific genes might reside outside previously annotated promoter regions. Analysis of high-coverage RNA-seq data in 43 MB samples revealed 262 novel first exons that were spliced to internal exons in excess of 15kb upstream of the previously annotated TSS, with some being located more than 500kb away[69]. Many of these alternate transcripts

were expressed in a subgroup-specific manner, which often coincided with patterns of differential DNA methylation in the region of the novel TSS. One notable example is the pluripotency factor *LIN28B*, which has been described to regulate multiple oncogenic processes including downregulation of the tumour-suppressive let-7 miRNA family[70]. In MB, *LIN28B* is expressed specifically in Group 3/4. In about half of Group 3 cases and nearly all Group 4 cases the annotated promoter region is fully hypermethylated, and transcription is initiated at a novel first exon that is spliced to the second annotated exon.

Systematic miRNA profiling of patient samples resulted in the identification of a number of differentially expressed miRNAs between MB subgroups and relative to normal controls[10,71,72]. Among the best studied is the oncogenic miR-17–92 cluster, which was described to be genetically amplified and over-expressed specifically in SHH-MB[71,73] and required for the formation of SHH-MB in the *Ptch1*$^{+/-}$ mouse MB model[74]. Furthermore, LNA-mediated silencing prolonged survival in intracranial SHH-MB allografts[75]. Another well studied example is the miR-183–96–182 cluster that is highly expressed in WNT- and Group 3/4-MB, targeting the AKT/PI3K/mTOR pathway and regulating cell proliferation and migration *in vitro* and *in vivo*[76–78]. Other miRNAs are downregulated in MB and have been described as tumour suppressors, including miR-125b, miR-326 and miR-324–5p, which have been described to regulate the SHH signaling pathway[79], and miR-124a and miR-9, regulating the REST complex and cell proliferation[80–83]. An extensive review on the role of miRNAs in MB has recently been published[84].

Genome-wide analysis of differential DNA methylation between MB subgroups and control tissues showed that the classical notion of gene silencing through promoter hypermethylation was not a prominent feature in MB[69]. The most abundant pattern of differential methylation was identified in regions extending several kilobases downstream of the promoter into the gene-body, in which hypomethylation correlated with elevated gene expression (promoter downstream correlated regions, pdCRs). About 20% of genes exhibiting MB subgroup-specific expression contained a pdCR, which suggests that this pattern plays an important regulatory role in distinct tumour-specific transcriptomes. Large, megabase-scale blocks of reduced DNA methylation (partially methylated domains, PMDs) represented another pattern of differential methylation in MB[69]. PMDs are a prominent feature in many cancer types and coincide with nuclear lamina-associated domains and other heterochromatic regions[85–87]. In MB, PMDs are primarily detected in the WNT and Group 3 subgroups and can cover up to one third of the genome, often in a subgroup-specific manner.

Integration of MB RNA-seq and enhancer ChIP-seq (namely H3K27ac) datasets has further enlightened mechanisms of gene regulation[88]. Enhancer ChIP-seq data generated for 28 primary MBs and 3 MB cell lines enabled annotation of the active cis-regulatory landscape across MB subgroups. In total, nearly 80,000 enhancers were inferred, 25% of which had not been previously annotated by ENCODE or the Roadmap Epigenomics Consortium. Computationally linking highly active, subgroup-specific enhancers, or super-enhancers (SEs), to putative target genes revealed new insights into the gene regulatory networks underlying MB subgroup biology and identity. Known cancer-associated genes were identified as prominent SE-targets, including ALK in WNT-MB, *GLI2*, *SMO*, and *NTRK3* in SHH-MB, *LMO1*, *LMO2*, and *MYC* in Group 3-MB, and *ETV4* and *PAX5* in

Group 4-MB, among others. Differential enhancer/gene target analysis identified aberrant TGF β signaling activity that was specific to Group 3, substantiating prior genomic-based evidence implicating oncogenic TGF β signaling in a subset of tumours[6].

Proteome

Proteins are the major functional product of the genome and dictate most cellular functions. Abundance, structure, stability, and post-translational modifications (PTMs) of proteins collectively increase the level of complexity and activity of the proteome. During the past decade, tremendous technical progress has been made towards deep detection and accurate quantification of the proteome[89] and recent studies have begun to explore the proteomic landscape of MB. Quantitative mass-spectrometry (MS)-based proteomics of primary human MBs predominantly confirmed the classification of MB into consensus subgroups in three independent cohorts[90–92]. The proteome also revealed notable substructure within both SHH (SHH-a and SHH-b) and Group 3 (Group 3a and 3b) subgroups; although these observations have been limited to modest sample cohorts and were not immediately supported by companion DNA methylation or transcriptomic datasets.

Concordance between mRNA transcript and protein abundance is known to vary between species and cell types, with only ~30–40% of protein variance explained by variance in mRNA abundance[93]. Group 3/4-MB exhibit the lowest correlation between mRNA and protein expression, emphasizing the potential role of post-transcriptional mechanisms in their underlying biology[91,92]. At the proteomic level, Group 3-MB exhibited elevated expression of eukaryotic initiation factor (eIF) subunits (EIF2s, EIF3s, EIF4Gs, and EIF4As)[49], a complex implicated in initiation of protein synthesis in eukaryotes. Supporting this finding, pharmacological inhibition of the formation of the eIE4F complex reduced MB cell viability *in vitro*[92]. Interestingly, discrepancies between mRNA and protein expression revealed activation of receptor tyrosine kinase (RTK) signaling in Group 4, especially aberrant expression of ERBB4 and phosphorylated SRC (Figure 4). *In utero* electroporation-mediated over-expression of activated SRC in combination with dominant-negative Tp53 induced Group 4-like MB *in vivo*[91], functionally substantiating observations gleamed through proteomics. While the mechanism(s) responsible for the mRNA/protein discrepancies seen in Group 3/4-MB are likely due to a multitude of factors, translational effects mediated through MYC or MYCN are suspected to play a role[91,94,95].

Phosphoproteomics can inform protein kinase activity and potential opportunities for therapeutic intervention through administration of pharmacological inhibitors. Bioinformatic analyses of MB phosphoproteomic data predicted activation of several prominent kinases, including GSK3 β (Group 4 and SHHb), PRKDC (Group 3 and WNT), CDK5 (Group 4) and CLK/CK2 (Group 3). Also, kinome analyses using high-throughput peptide phosphorylation profiling revealed two distinct protein-signaling signatures in MB: MYC-like protein-signaling observed in the majority of SHH and Group 3, and Protein-signaling profile-2, characterized by DNA-damage response, apoptotic, and neuronal signaling in the majority of Group 4[95]. Hence, these findings reinforce the idea that protein activity might reveal unifying and/or distinct tumour biologies amongst MB subgroups. Also, several PTMs of key players in MB have been identified. For instance, in SHH-MB, deacetylation

of Gli1 and Gli2 proteins induces their transcriptional activity[96], while phosphorylation of Atoh1 by Jak2 controls tumour growth[97]. Besides phosphorylation and acetylation, a myriad of other PTMs, including, ubiquitinylation, methylation and others have yet to be investigated in MB. The field of proteomics is rapidly advancing and along with further technological and bioinformatic breakthroughs, a deeper characterization of MB subgroup biology will undoubtedly follow.

Intratumoural heterogeneity

Phenotypic heterogeneity of individual cells within tumours has been a longstanding interest in MB research. In 2003, paralleled by similar discoveries in other solid tumours and leukemia[98,99], Peter Dirks and colleagues first reported the discovery of a stem-like tumour cell population in MB patient samples characterized by the neural stem cell surface marker CD133 (encoded by PROM1, positive for 6 to 21% of cells)[100,101]. These cells show marked capacity for proliferation, self-renewal, and neuronal differentiation both *in vitro* by neurosphere culture and *in vivo* by mouse xenotransplantation. As few as 1,000 CD133(+) cells were sufficient to initiate tumours in mice that phenotypically resemble the original tumour, whereas 50,000 CD133(−) cells failed to establish tumours. CD133 is most highly expressed in Group 3-MB[102], but does not mark tumour-propagating cells in SHH-MB GEM models[103,104]. Instead, the neural stem cell surface antigen CD15 was found to enrich for tumour propagating cells in this subgroup. Further analysis of the *Ptch1*$^{+/-}$ SHH-MB GEM model identified a rare, quiescent Sox2(+) cell population that gave rise to rapidly cycling progenitors[105].

In recent years, single-cell transcriptome sequencing (i.e. scRNA-seq) has emerged as a powerful method to decipher cellular states in healthy and diseased tissues in an unbiased way, as exemplified in adult and pediatric gliomas[106–110]. Recently, two independent studies applied scRNA-seq to cohorts of primary MBs, showing that MB displays subgroup-specific transcriptional heterogeneity at the single-cell level[111,112]. Analyzing eight patients from the SHH-, Group 3-, and Group 4-MB subgroups, Vladoiu and colleagues demonstrated mixed populations of cells with divergent differentiation along cerebellar neuronal lineages[111]. Analysing 25 patients across all molecular subgroups, Hovestadt and colleagues identified subgroup-specific undifferentiated and differentiated neuronal-like malignant populations[112] (Box 1). Both studies confirmed resemblance of SHH tumours to the cerebellar granule neuron lineage, in agreement with earlier experimental evidence[9,113] (Figure 5; see Box 1). Interestingly, adult SHH-MB tumours showed a higher fraction of undifferentiated granule neuron progenitors (GNPs) compared to infant tumours, possibly linking to their divergent biology (Box 1). Combined analysis of Group 3/4-MB tumours revealed a related developmental trajectory from primitive progenitor-like to more mature neuronal-like cells, whose relative proportions distinguished these subgroups (Box 1). *MYC*-amplified Group 3-MBs only comprised undifferentiated progenitor-like cells and did not show any capacity to differentiate. Most other Group 3-MBs showed a small degree (<10%) of neuronal differentiation, indicating that tumour cells maintained the capacity to differentiate. Interestingly, a subset of tumours characterized as ‘intermediate’ cases by DNA methylation-based classification exhibited both undifferentiated and differentiated populations at varying proportions, providing an explanation for the challenges to

confidently assign some Group 3/4-MB cases to either subgroup by bulk molecular profiling (Box 1). Prototypic Group 4-MBs were comprised almost exclusively of more differentiated neuronal-like cells resembling unipolar brush cells (UBCs) and glutamatergic cerebellar nuclei (GluCN) (Figure 5; see Box 1).

By performing parallel gene expression profiling and exome sequencing of 47 multiregional biopsies from eight patients, it was recently concluded that MB is characterized by spatially homogeneous transcriptomes, in which biopsies from the same patient were more similar to each other than to those of other patients[114]. This was contrary to findings in glioblastoma, in which different biopsies from the same patient classified as different transcriptional subtypes[114]. However, the same study reports high levels of genetic heterogeneity in MB at the level of SCNAs and somatic mutations. As affected genes included therapeutic targets, these observations put the representativity of single biopsies and the efficacy of monotherapies to treat MB into question.

Molecular insights into MB relapse and metastasis

Since MB relapse and metastases remain the most significant morbidity factors influencing patient outcomes[115], an improved understanding of the molecular events driving treatment resistance and recurrence represents a major priority. To date, comparative studies of primary versus relapse disease have been exceedingly limited and restricted to relatively modest cohorts. Application of a NanoString-based molecular classification approach confirmed that MB subgroup status is conserved between primary and relapse disease, reinforcing the concept that individual MB subgroups constitute distinct diseases[116]. This observation was later independently confirmed using a complementary subgrouping approach[117]. Specific emergence of *MYC/MYCN* amplifications and TP53 pathway defects (namely *TP53* mutation, *CDKN2A* deletion) at relapse was also reported[117]. By way of an elegant *Sleeping Beauty* transposon-based murine model of SHH-MB (*Ptch1*$^{+/-}$/*Math1-SB11/T2Onc or T2Onc2*), compelling evidence for genetic divergence of primary versus recurrent tumours was substantiated by a paucity of shared genetic events between treatment-naïve and recurrent mouse MBs[118]. In the same study, a cohort of 36 primary/relapse MB patient tumours were subjected to genomic characterization. Recurrent tumours harbored a profound increase in mutational burden and SVs, the overwhelming majority of which were restricted to either primary or relapse disease, with minimal overlap observed between both disease compartments. More extensive efforts that are sufficiently powered to build on these studies and provide further understanding of the molecular basis of MB treatment failure and relapse are ongoing.

MB metastasis almost always occurs via leptomeningeal dissemination (LMD) that remains confined to the CNS and spinal cord[115]. Approximately one third of all MB patients are metastatic at diagnosis, with patterns and frequencies that vary considerably according to molecular subgroup[119]. Patients that fail conventional therapy and/or relapse metastatically share a universally dismal prognosis, with virtually all patients succumbing to their refractory disease. As such, there is a critical and urgent need to decipher the molecular and cellular mechanisms governing LMD in this unacceptably high fraction of affected MB patients. Through genomic analysis of simultaneously collected patient-matched primary

MB, leptomeningeal metastatic MB, and peripheral blood, Garzia and colleagues inferred the presence of circulating tumour cells (CTCs) based on careful analysis of specific mutations that were clonal in the metastasis, clonal or subclonal in the primary tumour, and present at very low fractions in the peripheral blood[120]. In 3/6 of the evaluated peripheral blood samples, exceedingly rare CD56(+)/CD45(−) (both of which were used to mark malignant cells) morphologically abnormal cells were identified, further supporting the presence of rare CTCs in the blood of some MB patients. These molecular and cellular observations derived from patient samples were strengthened by a series of innovative mouse modeling experiments, including implementation of a parabiosis model that established hematogenous spread of implanted MB cells between surgically connected donor (implanted mouse) and recipient (sibling mouse) animals. Comparison of gene expression profiles for a limited set of patient-matched primary and metastatic MB samples in the same study identified over-expression of the chemokine *CCL2* in the metastatic compartment, that was further substantiated through additional molecular and functional analyses, suggesting that aberrant CCL2 signaling may be an important mediator of LMD in MB.

Emerging clinical implications & translational opportunities

Risk stratification.

Clinically relevant insights uncovered during the molecular era have enabled cautious, yet steady transition of discoveries made in the research arena into the clinical setting. Indeed, clinical protocols for MB patients have begun to implement molecular subgroup-informed strategies for treatment stratification. SJMB12 (NCT01878617), an active trial for newly diagnosed MB patients includes separate treatment arms for WNT, SHH, and non-WNT/non-SHH patients (i.e. Group 3 and Group 4). On this protocol, clinically standard-risk WNT-MB patients (non-metastatic with near total surgical tumour resection) receive reduced craniospinal radiation (CSI; 15Gy as opposed to the standard dose of 23.4Gy administered to standard-risk MB patients), owing to the highly favorable outcomes that have been consistently reported for WNT-MB since 2005[28,121]. Skeletally mature (i.e. females with a bone age ≥15 years or males with a bone age ≥17 years) SHH-MB patients enrolled on SJMB12 receive the SMO inhibitor, vismodegib, on top of standard of care chemotherapy and CSI. In contrast, non-WNT/non-SHH patients are further stratified into standard- and high-risk treatment arms based on a combination of clinical- and molecular-risk factors, including extent of resection, metastatic status, and *MYC* amplification status. A trial for newly diagnosed patients ongoing in Europe, International Society of Paediatric Oncology (SIOP) PNET 5 (NCT02066220), is likewise stratifying WNT-MB patients according to a low-risk treatment arm and administering reduced dose CSI (18Gy) compared to non-WNT-MB patients that are treated according to standard of care; an effort that is also being mirrored for WNT-MB patients by Children's Oncology Group (ACNS1422; NCT02724579) in North America. Despite these examples of molecularly-informed risk-stratification in current MB trials, the clinical arena still lags behind the pace at which retrospective research studies enlighten new concepts. Indeed, recent molecular studies conducted on both large retrospective and trial cohorts have provided increased rationale for

further molecularly-driven stratification in future protocols, especially within specific MB subgroups[25,27,31,38,122].

Genetic predisposition.

Hereditary genetic predisposition to MB remains an underappreciated clinical challenge. Recent data collected on large retrospective MB cohorts suggests that especially in the case of SHH-MB, the proportion of patients with underlying cancer predisposition might approach ~25% or even higher[29]. In WNT-MB patients, the proportion appears to be in the range of 5–10%. At this stage, these estimates only account for known cancer predisposition genes and efforts to systematically analyze yet unknown pathogenic events in the germline of MB patients are currently ongoing. Based on published AACR guidelines[123] that suggest genetic testing and counseling for specific tumour types that frequently occur in the context of genetic predisposition (threshold of ≥10%), the prevalence of clearly pathogenic germline events in known cancer predisposition genes certainly qualifies this disease (especially SHH-MB, but also WNT-MB) for a general recommendation to offer genetic testing and counselling prior to adjuvant therapy. This infrastructure, however, is currently not in place in the majority of treatment centers across the world. The largest MB predisposition study to date[29] identified *APC* germline mutations in *CTNNB1* mutation-negative WNT-MB patients, as well as significant enrichment of *TP53*, *SUFU*, *PTCH1*, *BRCA2* and *PALB2* pathogenic germline variants in SHH-MB. These genes can be tested at once by clinical grade whole-exome or panel sequencing, but also successively as single gene tests based on their age associations and, in some cases, family history. AACR surveillance guidelines are available for *APC*, *TP53*, *SUFU*, and *PTCH1*, whereas it remains to be determined how to appropriately manage MB patients with damaging heterozygous germline variants in *BRCA2* and *PALB2*. Since this considerable proportion of MB patients with hereditary disease and their families require special clinical attention including potential treatment modifications, family testing, and surveillance, this challenge has to be tackled systematically, but expeditiously.

Therapeutic targets.

Arguably it was the discovery of small molecule hedgehog pathway antagonists (i.e. SMO inhibitors) and their preclinical potency against *Ptch1*$^{+/-}$ GEM models[124] that sparked the need to molecularly identify MB patients that would benefit from these inhibitors. However, what emerged was molecularly far more complicated than first envisioned. SHH pathway gene alterations in SHH-MB differ according to patient age at diagnosis and subtype[25,26,32,38], collectively accounting for the variable responses to SMO inhibitors. *PTCH1* mutations (both germline and somatic) are the most common but present in less than half of all SHH-MB patients and mostly within infants and adults. Germline and somatic *SUFU* mutations are largely restricted to infant SHH-MBs, whereas activating somatic *SMO* mutations are enriched in adult SHH-MB patients. Similarly, germline and somatic *TP53* mutations, predominantly coincident with *GLI2* and *MYCN* amplifications, are exclusively found in children between the ages of 8–17 years old. These observations are of direct clinical relevance when considering treatment of SHH-MB patients with SMO inhibitors. Treatment of the *Ptch1*$^{+/-}$ GEM model[124] and SHH-MB patient derived xenograft (PDX) model[32] harboring *PTCH1* mutations were determined to be sensitive to the SMO

inhibitors (Hhantag, vismodegib, sonidegib), whereas a GEM model lacking *Sufu*[125] or PDX harboring *TP53* mutation and *MYCN* amplification exhibited primary resistance[32]. These preclinical findings were corroborated clinically in an early phase clinical trial on patients with relapsed SHH-MB when treated with the SMO inhibitor, vismodegib[126]. Once again, as molecularly predicted, responders to SMO inhibition were more likely to have tumours that harbored a *PTCH1* mutation whereas no beneficial clinical responses were observed in patients harboring *TP53* or *SUFU* mutations and concurrent *MYCN* and *GLI2* amplifications. Moreover, another significant concern that has restricted the clinical use of SMO inhibitors is the emergence of permanent growth plate fusions in the long bones of small children[127], a morbidity that was predicted in early preclinical studies on young mice[128]. Collectively, these findings demonstrate the importance of performing thorough preclinical and molecular testing to identify the appropriate patient populations as candidates for molecularly targeted therapy.

Mechanistically, GFI1 was recently shown to mitigate its oncogenicity in MB through direct interaction with KDM1A/LSD1[129], a histone lysine demethylase associated with transcriptional repression. Treatment of MYC/GFI1-driven MBs with LSD1 inhibitors attenuated the malignant phenotype *in vitro* and *in vivo* using a flank PDX model. No treatment effect was observed when treating the same tumours in an orthotopic setting, demonstrating that this model might serve as a preclinical testing vehicle for blood-brain-/blood-tumour-barrier penetration. Taken together, these results provide preclinical support for treating affected GFI1/GFI1B-driven MB patients with LSD1 inhibitors, given that a brain penetrant LSD1 inhibitor will eventually become available.

With the identification of aberrant TGF β signaling in Group 3-MB and RTK signaling in Group 4-MB, new opportunities for preclinical testing of inhibitors to these pathways have likewise emerged[91,130]. Additional studies evaluating the efficacy of such agents in treating accurate preclinical MB models will be necessary to further substantiate their future implementation in the clinic.

Conclusion and future outlook

Through continued multi-omic analyses conducted on unprecedented patient cohorts, MB now undoubtedly represents one of the most extensively characterized cancer entities. Deep understanding of molecular substructure and assignment of known and novel driver gene alterations to specific disease subtypes has created a more refined understanding of tumour biology. This knowledge will enable the development of better models that more accurately recapitulate what is seen in patients. Moreover, these advances pave the way for critical functional studies required to determine the mechanistic contribution of newly discovered genes and molecular complexes to MB pathogenesis, in the appropriate cellular context. A continued transition from bulk tumour profiling to more detailed analyses of intratumoural heterogeneity in single-cells is to be expected, especially as methods for analyzing genetic alterations, the epigenome, transcriptome, and proteome at single-cell resolution continue to evolve and become attainable on archival clinical samples. These studies will prove essential for further resolving recently described molecular subtypes within subgroups, elucidating the molecular and cellular basis of MB recurrence and metastasis, and more.

Clinically, the separation of MB along molecular lines into subgroups and now into subtypes within subgroups generates concern among many treating physicians that the number of divisions far outpaces our ability to give subtype-specific care. However, while this argument against a complicated classification system is understandable given that the majority of subtype-specific findings are not immediately targetable by individual medicines, it ignores the clinical benefit of improved risk stratification. Despite reasonably high cure rates in the range of 70–75% for all, the cost of current therapy that combines surgery, CSI, and chemotherapy remains unacceptably high. Molecular subgrouping has already demonstrated pronounced differences in survival, and this has afforded the opportunity to trial judicious reductions in therapy to the favorable WNT subgroup while maintaining and optimizing intensive therapy for the unfavorable Group 3 patients. Moreover, the more precise and more well defined the subtypes become the better the opportunity to hone this therapeutic approach. For example, the new subtyping of very young children with MB is anticipated to fairly rapidly lead to new trials since the toxicities of CSI and chemotherapy remain so unacceptably high in this most vulnerable population. Furthermore, if promising targeted therapies such as SMO inhibitors, LSD1 inhibitors, and RTK inhibitors are to be successful, then it is imperative that these agents are given to the appropriately targeted populations. Even though the end of further evolution of MB molecular sub-classification may be in sight, the impact of these molecularly informed advances on therapy is only just beginning.

MB subgroup origins (Box 1)

As MB subgroups are enriched for specific genetic alterations and exhibit discriminatory epigenetic and transcriptional profiles, it has long been suspected that they arise from distinct cellular populations or developmental lineages[131]. Mouse models of the WNT and SHH subgroups have respectively substantiated lower rhombic lip progenitors (Blbp+) and GNP populations (Atoh1+) as probable cells-of-origin for these subgroups[132]. Multiple orthotopic and somatic gene transfer models have demonstrated that a variety of progenitor cell populations can be effectively transformed to replicate molecular and phenotypic features of Myc-driven Group 3-MB[133–135]. Mapping the MB enhancer landscape enabled inference of master transcription factors (TFs) governing subgroup-specific tumour biology. LMX1A, EOMES, and LHX2 were predicted to function as master regulators of Group 4-MB. Developmentally, these master TFs regulate lineage specification for restricted glutamatergic progenitor cell populations born out of the cerebellar upper rhombic lip during cerebellar morphogenesis, including glutamatergic cerebellar nuclei (GluCN; also known as deep cerebellar nuclei; DCN) and unipolar brush cells (UBCs). Since LMX1A, EOMES, and LHX2 were inferred to be highly specific SE-regulated candidate master TFs in Group 4-MB, the aforementioned glutamatergic populations expressing these markers were proposed as the putative lineage(s)-of-origin for this MB subgroup. More recently, comparative cross-species transcriptomic analyses have complemented these observations, utilizing single-cell transcriptional profiles of the developing mouse cerebellum as a reference for mapping MB subgroup origins[111,112]. Granule neuron lineage populations were shown to be highly correlated with SHH-MBs, reinforcing the expansive literature implicating GNPs as their developmental origin. Interestingly, GluCN and UBCs were both shown to be highly

transcriptionally similar to Group 4-MBs, suggesting that these populations could represent *bona fide* cells-of-origin for this subgroup of patients. Early embryonic Nestin+ progenitor cells were suggested as being highly correlated with Group 3-MB[111]; although, these results were not supported in the companion study[112]. Deeper molecular analyses on these novel candidate populations and functional studies that mimic relevant candidate driver alterations in the correct lineage at the correct developmental stage are ongoing and will be required to further substantiate these initial findings.

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Glossary

Next-generation sequencing

Technologies enabling massively parallelized reading of amplified short nucleotide sequences (typically yielding hundreds of million reads 100–500 bp in length). Broad availability of these technologies has transformed genomic research in the last decade. In contrast, emerging third-generation sequencing technologies read sequences without prior amplification, yielding much longer reads, albeit with reduced accuracy and throughput.

Molecular classification

Classification of patient tumour samples based on molecular markers, opposed to classification based on histomorphological appearance. Commonly used molecular markers in MB include genetic mutations (e.g. mutations in *CTNNB1* in WNT-MB), copy-number alterations (e.g. *MYC* amplification in Group 3-MB), and genome-wide gene expression and DNA methylation patterns for classification into molecular subgroups and subtypes.

Intratumoural heterogeneity

Observation that tumours comprise of distinct malignant and non-malignant (cells of the micro-environment) cell types. Heterogeneity of malignant cells encompasses genetic heterogeneity (e.g. different genetic subclones) and transcriptional heterogeneity (e.g. malignant cell states resembling normal development).

DNA methylation

A central epigenetic mark in which a methyl group is added to cytosine bases in genomic DNA. Cytosine methylation is inherited through cell division and plays important roles in gene regulation during normal human development and diseases such as cancer. Genome-wide methylation patterns constitute very stable and informative molecular markers for tumour subgroup and subtype classification.

Isochromosome

An abnormal chromosome in which both chromosome arms are identical. Isochromosome 17q is one of the most frequent copy-number alterations in Group 3/4-MB, in which a second q-arm is fused to the p-arm proximal to the centromere in most cases.

Non-synonymous mutation

A genetic alteration that alters the amino acid sequence of an affected protein, possibly altering protein function. Most described recurrent mutations in MB are non-synonymous mutations. Other types of mutations include synonymous mutations (silent substitutions), which do not alter the protein sequence because of the degeneracy of the genetic code, and non-coding mutations, which affect regions that are not translated into proteins. Both synonymous mutations and non-coding mutations can have important gene regulatory functions. The most common non-coding mutation in MB are found in a hotspot in the promoter of the *TERT* gene.

Chromatin

Describes the complex of genomic DNA and proteins. Its main functions are packaging of DNA and regulation of gene expression. The main type of proteins in chromatin are histones. Histone tails are frequently marked by post-translational modifications that mediate changes in chromatin structure and accessibility through interaction with histone modifiers and/or nucleosome remodeling complexes. Genome-wide patterns of histone modifications can be analyzed by chromatin immunoprecipitation-coupled sequencing (ChIP-seq).

Germline mutation

Describes genetic variation within the germ cells of a carrier that can be passed on to offspring. If inherited, this variation is then present in all somatic and germline cells of the offspring (constitutional mutation). Germline mutations can predispose to cancer. Common examples of germline mutations associated with MB include mutations of *PTCH1* (Gorlin syndrome) and *TP53* (Li-Fraumeni syndrome).

Patient derived xenograft

Models of cancer in which tumour cells from a patient are implanted and maintained in a non-human carrier, most commonly immunodeficient or humanized laboratory mice. PDX models are thought to be more closely resembling patient tumours than cell cultures. Orthotopic models describe models in which cells are implanted in the corresponding anatomical position, compared to heterotopic models in which cells are implanted in an area unrelated to the original tumour site (e.g. subcutaneously).

Structural variation

Describes genomic variations such as deletions, duplications, inversions, and translocations that affect the structure the chromosome at the microscopic and submicroscopic level. Structural variants can result in fusion genes and enhancer hijacking. An extreme form of structural variation observed in MB is a phenomenon termed chromothripsis, in which a large number of rearrangements affect a single chromosome.

Blood-brain barrier

Is a semipermeable border formed by endothelial cells lining the cerebral microvasculature that separates the brain from the circulating blood. The blood-brain barrier thereby protects the brain from fluctuations in plasma composition and circulating agents such as neurotransmitters and pathogens. The blood-brain barrier also presents a challenge for drug delivery when treating brain tumours.

microRNA
An abundant class of small non-coding RNA molecules that are approx. 21–22 nucleotides in length. In complex with the RNA-induced silencing complex these molecules exert important gene regulatory functions by post-transcriptional gene silencing through base complementarity to target genes.

CRISPR gene editing
A novel method that allows for efficient genetic manipulation to study the effect of coding and non-coding variants in model systems. Delivery of the CRISPR-Cas9 nuclease complex is guided by base complementarity of synthetic guide RNAs to genomic regions.

Glutamatergic neurons
A class of neurons that release the excitatory neurotransmitter glutamate. The second major class of neurons are GABAergic neurons that release the inhibitory neurotransmitter GABA (gamma-aminobutyric acid).

Mass-spectrometry
An analytical technique that allows accurate mass determination of large biomolecules such as proteins. Important applications of mass-spectrometry are the quantification of protein expression levels and the analysis of post-translational protein modifications (PTMs).

Single-cell RNA-seq
Emerging technology that enables unsupervised characterization of transcriptional profiles in individual cells of healthy and diseased tissues. Throughput of technologies has steadily increased over recent years, now enabling profiling of tens-of-thousands of individual cells in a single experiment.

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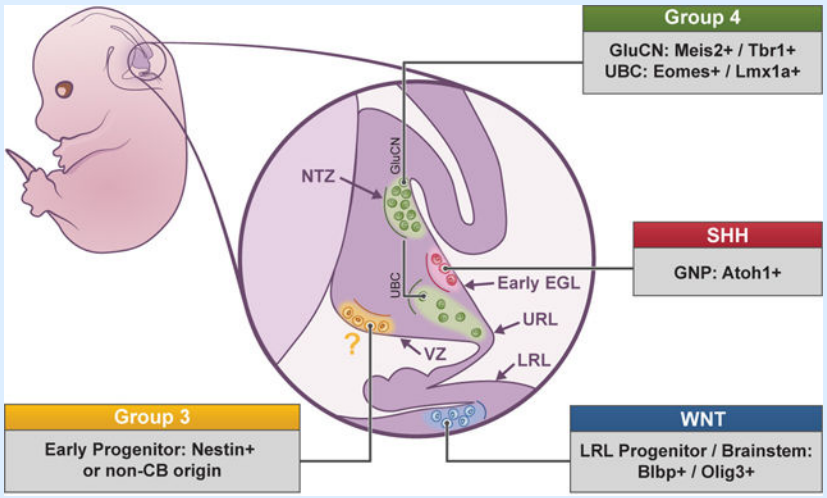
Box 1 | Mapping cellular origins of MB subgroups.

Graphical depiction of an embryonic mouse cerebellum (estimating development at ~13.5) highlighting cellular populations proposed to be developmentally linked to specific MB subgroups. GluCN, glutamatergic cerebellar nuclei; UBC, unipolar brush cell; GNP, granule neuron progenitor; LRL, lower rhombic lip; CB, cerebellar; URL, upper rhombic lip; EGL, external granule layer; VZ, ventricular zone; NTZ, nuclear transitory zone.



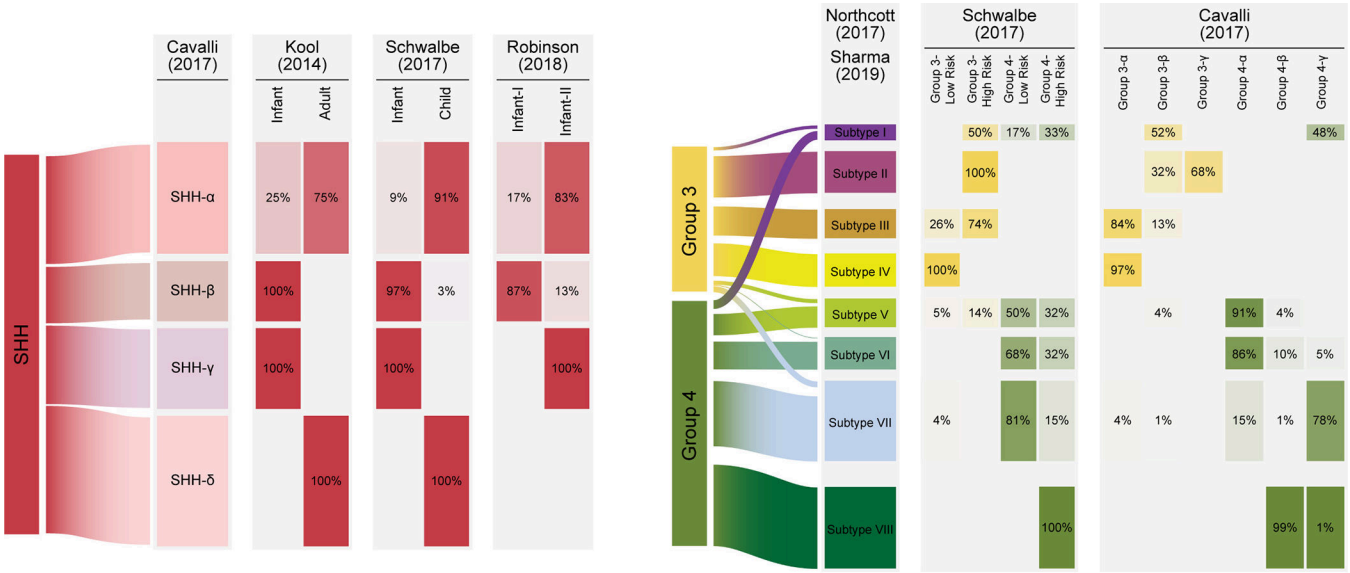


Figure 1 |. Comparison of MB DNA methylation-derived subtypes described across recent studies.

a | Correspondence between four molecular subtypes of the SHH-MB subgroup described by Cavalli et al. and the subtypes described in three additional studies. DNA methylation profiles from all samples of each additional study were used to classify patients into the four molecular subtypes using a machine learning approach. The height of each row corresponds to the fraction of samples per subtype in the Cavalli et al. study. Percentages indicate overlap of predicted subtypes with original subtype annotations in each additional study. No samples from the study by Robinson et al. predicted as SHH-δ, because the study only included patients under the age of six years.

b | Similar comparison between the eight molecular subtypes of Group 3/4-MB described by Northcott et al. and Sharma et al. and the subtypes described in two additional studies. Line width between consensus subgroups and DNA methylation subtypes indicate the fraction of samples per subtype originally classified as Group 3- or Group 4-MB.

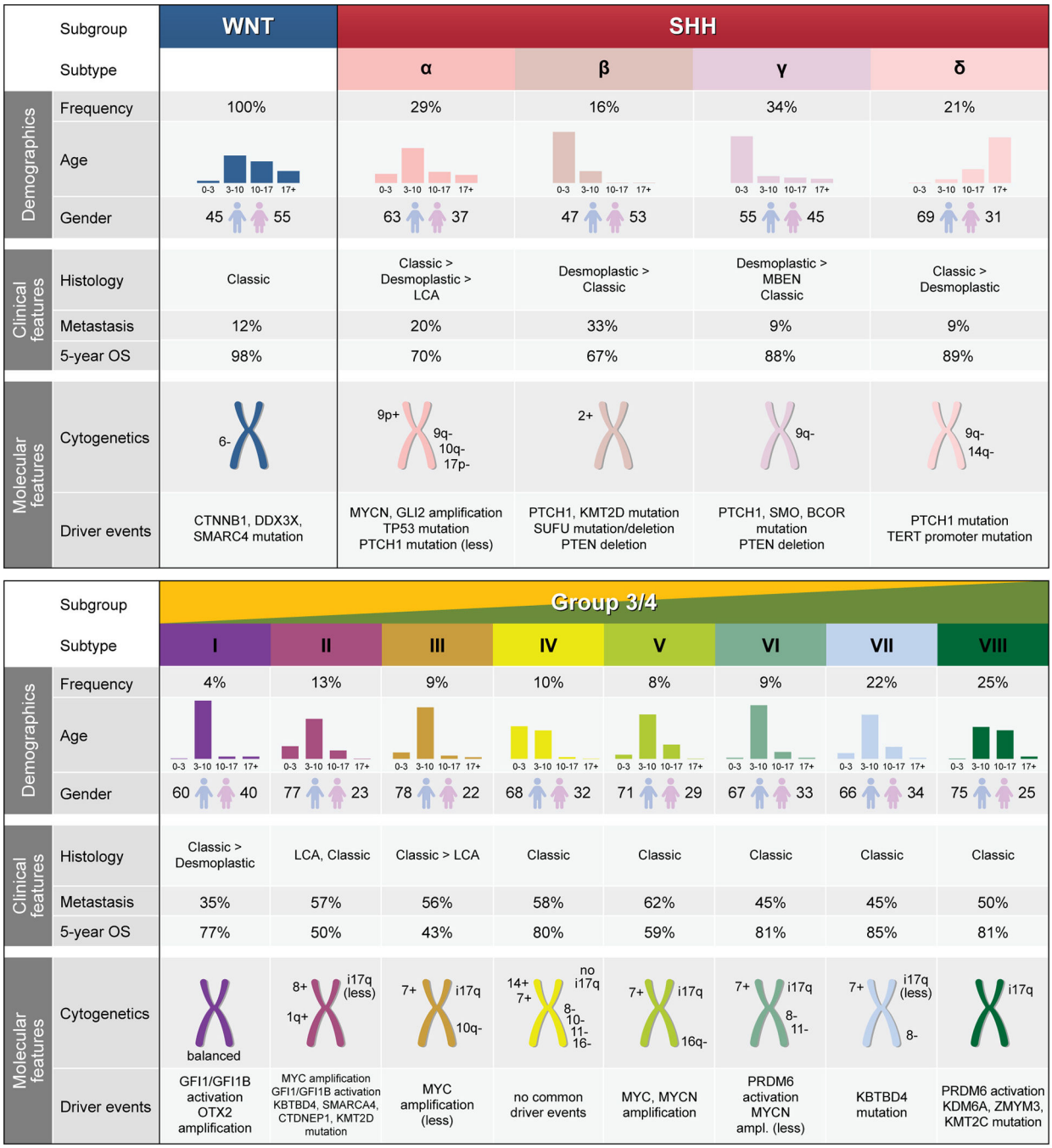


Figure 2 |. Summary of demographic, clinical, and molecular features of novel MB subtypes.

Values for age and gender distribution, frequency of metastasis, and 5-year overall survival for the WNT and SHH subgroups are derived from the Cavalli et al. study. Driver events were additionally derived from the Kool et al. and Robinson et al. studies. Similarly, values for the Group 3 and Group 4 subgroups were derived from the Sharma et al. study. OS, overall survival; LCA, large cell anaplastic; MBEN, medulloblastoma with extensive nodularity; i17q, isochromosome 17q; BCOR, BCL6 Corepressor; CTDNEP1, CTD Nuclear Envelope Phosphatase 1; CTNNB1, Catenin Beta 1; DDX3X, DEAD-Box Helicase 3 X-

Linked; GFI1, Growth Factor Independent 1 Transcriptional Repressor; GFI1B, Growth Factor Independent 1B Transcriptional Repressor; GLI2, GLI Family Zinc Finger 2; KBTBD4, Kelch Repeat and BTB Domain Containing 4; KDM6A, Lysine Demethylase 6A (UTX); KMT2C, Lysine Methyltransferase 2C (MLL3); KMT2D, Lysine Methyltransferase 2D (MLL2); MYC, MYC Proto-Oncogene BHLH Transcription Factor; MYCN, MYCN Proto-Oncogene BHLH Transcription Factor; OTX2, Orthodenticle Homeobox 2; PRDM6, PR/SET Domain 6; PTCH1, Patched 1; PTEN, Phosphatase and Tensin Homolog; SMARCA4, SWI/SNF Related Matrix Associated Actin Dependent Regulator of Chromatin Subfamily A Member 4; SMO, Smoothened Frizzled Class Receptor; SUFU, SUFU Negative Regulator of Hedgehog Signaling; TERT, Telomerase Reverse Transcriptase; TP53, Tumour Protein P53; ZMYM3, Zinc Finger MYM-Type Containing 3.

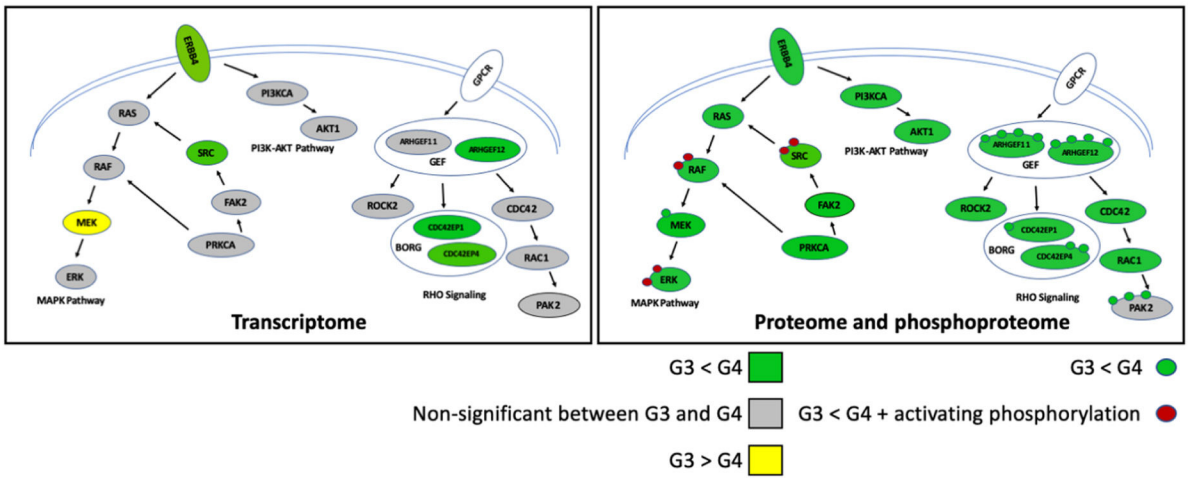


Figure 3 |. Recurrently altered genes and pathways across MB subgroups.

Grouped barplot of recurrently altered genes identified in WNT- (blue), SHH- (red), Group 3- (yellow) and Group 4-MB (green). Altered genes are grouped by functional categories. The percentage of affected samples is indicated (y-axis). Mutations of *CTNNB1* are identified in 85.7% of WNT-MB.

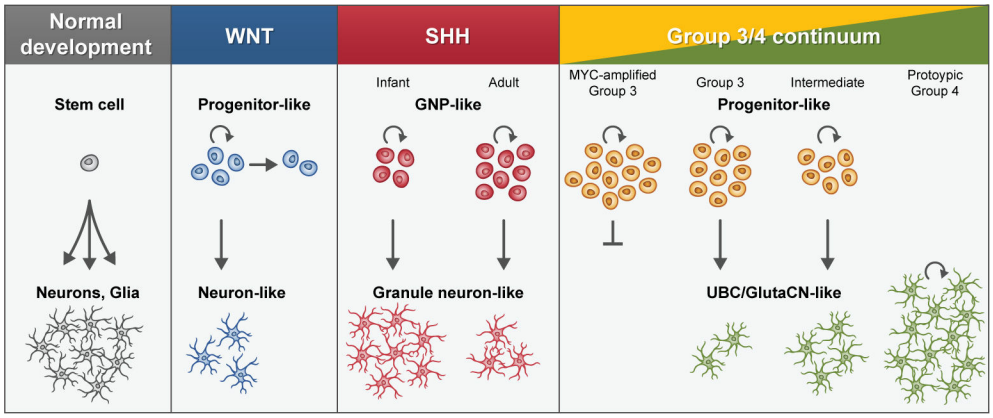


Figure 4 |. Aberrant signaling pathways implicated in Group 3 and Group 4 MB by proteomics. Colors indicate the relative abundance in mRNA (left part), protein and phosphoprotein (right part) between Group 4 and Group 3. Phosphorylation is indicated as a small circle. The half circle indicates the plasma membrane.