Hijacking a neurodevelopmental epigenomic program in metastatic dissemination of medulloblastoma

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Abstract

How dysregulation of neurodevelopment relates to medulloblastoma (MB), the most common pediatric brain tumor, remains elusive. Here, we uncovered a neurodevelopmental epigenomic program being hijacked to induce MB metastatic dissemination. Unsupervised analyses by integrating publicly available datasets with our newly generated data revealed that SMARCD3/BAF60C regulates DAB1-mediated Reelin signaling in Purkinje cell migration and MB metastasis by orchestrating cis-regulatory elements (CREs) at the DAB1 locus. We further identified that a core set of transcription factors, enhancer of zeste homolog 2 (EZH2) and nuclear factor I X (NFIX), coordinates with the CREs at the SMARCD3 locus to form a chromatin hub for controlling SMARCD3 expression in the developing cerebellum and metastatic MB. Elevated SMARCD3 activates Reelin/DAB1-mediated Src kinase signaling, resulting in MB response to Src inhibition. These data deepen our understanding of how neurodevelopmental programming influences disease progression and provide a potential therapeutic option for MB patients.

Main

The development of an organism is a precisely orchestrated temporal and spatial process, in which dysregulation of every biological factor may be related to diseases, such as medulloblastoma (MB), the most common brain cancer of childhood. MB is classified as an embryonal tumor arising in the cerebellum and causes a high rate of morbidity and mortality in children. Molecular characterization of MB revealed the disease heterogeneity associated with four major subgroups, WNT, SHH, Group 3, and Group 4. Notably, Group 3 MB (G3 hereafter), accounting for 25%-30% of all MBs, is the most aggressive and malignant, characterized by frequent metastasis at diagnosis and the worst prognosis. While surgical resection, radiation, and chemotherapy are effective at eliminating some forms of MBs, patients with high-risk tumors (e.g., G3) are more likely to suffer disease progression after initial therapy.

Metastatic tumors, rather than primary tumors or recurrent tumors at the primary sites, have a particularly high mortality rate in MB patients. Despite rarely spreading to extraneural organs, MB metastasizes almost exclusively to the spinal and intracranial leptomeninges through the cerebrospinal fluid and/or the bloodstream. However, how MB cells acquire the capability of mobility for metastatic dissemination is poorly understood.

G3 is thought to arise from Nestin* early neural stem cells that give rise to GABAergic and glutamatergic neurons, the two major lineages of the cerebellum. Over the past decades, the morphological, cellular, and molecular features of the developing cerebellum have been extensively explored, implicating that abnormal cerebellar development is a major determining factor for neurological diseases, including MB. Although MB is linked to aberrant cerebellar development, cellular and molecular mechanisms of tumor metastatic dissemination remain elusive.

In this study, we identified a novel molecular circuit to regulate the migration and positioning of Purkinje cells (PCs), a principal GABAergic neuron in cerebellar development. Interestingly, MB hijacks this molecular circuit using an abnormal epigenetic program to promote tumor metastatic dissemination. These findings shed light on the mechanisms associated with tumor dissemination and potential new targeted therapies for this devastating brain cancer in children.
Results

SMARCD3 expression is elevated in G3 and associated with tumor metastasis

Given that epigenetic deregulation plays a critical role in the development and progression of MB\textsuperscript{14}, we explored epigenetic regulators involved in the oncobiology of G3. We first defined G3-associated differentially expressed genes (DEGs) by analyzing transcriptomic data from 1,350 patient MB and 291 normal cerebellum samples\textsuperscript{15} (Fig. 1a). Second, the G3-associated DEGs were intersected with epigenetic-related genes from the EpiFactors database containing 720 DNA/RNA-, histone-, and chromatin-modifying enzymes and their cofactors\textsuperscript{16}. Surprisingly, \textit{SMARCD3} was the sole G3-associated DEG related to epigenetic modifications (Fig. 1b). Analysis of two transcriptomic datasets\textsuperscript{15,17} revealed that \textit{SMARCD3} mRNA expression levels were significantly higher in G3 than those in other MB subgroups and normal tissues (Fig. 1c and Extended Data Fig. 1a). Analysis of single-cell RNA sequencing (scRNAseq) data\textsuperscript{18} demonstrated that the majority of G3 cells (40.98\%) expressed \textit{SMARCD3} compared with cells in other subgroups (G4: 15.67\%; SHH: 5.43\%; WNT: 13.14\%) (Fig. 1d and Extended Data Fig. 1b). Consistently, higher levels of SMARCD3 protein expression were observed in G3 compared with other MB subgroups in a proteomic dataset\textsuperscript{19} (Fig. 1e). Higher levels of \textit{SMARCD3} mRNA expression were significantly correlated with poorer prognosis of MB patients, which was independent of age and sex (Fig. 1f and Extended Data Fig. 1c). Immunohistochemistry (IHC) analysis using human MB tissue microarrays revealed that high levels of SMARCD3 protein were also associated with worse patient outcomes (Fig. 1g). These results suggest that SMARCD3 may play a critical role in G3 development and progression.

To determine SMARCD3 functions, we performed gene ontology (GO) analysis based on SMARCD3-associated genes in MB using a transcriptomics dataset\textsuperscript{4} (Supplementary Table 1) and identified that SMARCD3 was involved in biological processes for regulating cell membrane projection and organization related to cell motility and migration (Fig. 1h). Thus, we hypothesized a positive correlation between high levels of SMARCD3 expression and increased tumor metastasis. To this end, analysis of transcriptomic and proteomic datasets\textsuperscript{4,19} revealed that patients with metastases from all MB subgroups and G3 only exhibited higher levels of SMARCD3 mRNA and protein expression than those in patients without metastases (Fig. 1i and Extended Data Fig. 1d), respectively. Consistently, patients with higher SMARCD3 levels had a higher frequency of tumor metastasis (Extended Data Fig. 1e, f). Experimentally, G3 cell lines with higher SMARCD3 expression levels exhibited increased migratory abilities in transwell assay and a higher metastatic capacity in the brain and spine of xenograft MB mice (Fig. 1j, k, and Extended Data Fig. 1g). Together, these data demonstrate a strong correlation between SMARCD3 expression and tumor migration and metastasis in MB.

\textbf{SMARCD3 drives MB cell migration and tumor metastatic dissemination}

To examine if SMARCD3 promotes MB cell migration \textit{in vitro} and \textit{in vivo}, we generated CRISPR/Cas9-mediated SMARCD3 knockout (KO) G3 cell lines and found that SMARCD3 deletion significantly decreased cell migration in MED8A and D341 cells by scratch-wound healing and transwell assays (Fig. 2a, b, and Extended Data Fig. 2a-d). Bioluminescence imaging (BLI) of orthotopic xenograft mice bearing MED8A with SMARCD3 KO showed a decreasing percentage of spinal metastasis compared with control (WT) (Fig. 2c and Extended Data Fig. 2e). Notably, we observed that SMARCD3 was highly expressed in the tumor margin compared with the tumor center (Fig. 2d), suggesting that MB cells with high levels of SMARCD3 tend to spread from the primary tumor site.
Of note, SMARCD3 expression levels in the metastatic tumor cell line D458 were higher than those in the matched primary tumor cell line D425\textsuperscript{20} (Fig. 1j). To further test the SMARCD3 function in determining MB metastatic dissemination, we performed loss- and gain-of-function studies using these paired cell lines. SMARCD3 deletion significantly decreased D458 cell migration and spinal metastasis in orthotopic xenograft mice (Fig. 2e, f, Extended Data Fig. 2f, g). Circulating tumor cells (CTCs) in peripheral blood are considered to mediate MB leptomeningeal metastasis\textsuperscript{6}. Therefore, we generated the orthotopic xenograft mice bearing GFP-labeled D458 cells with SMARCD3 KO or WT (Fig. 2g) and observed fewer mice with CTCs (at least more than 1 GFP\textsuperscript{+} cell in 10,000 total peripheral blood mononuclear cells) after SMARCD3 deletion (Fig. 2h). Conversely, overexpression (OE) of SMARCD3 in D425 significantly increased cell migration, spinal metastasis, and the percentage of tumor-bearing mice with CTCs (Fig. 2i-k, Extended Data Fig. 2h, i). Moreover, SMARCD3-enhanced tumor dissemination was visualized in the local brain cortex and the spinal cord by assessing D425 (WT vs SMARCD3 OE)-derived GFP\textsuperscript{+} xenograft mice (Fig. 2l, m). These results suggest a pivotal role of SMARCD3 in the phenotypic determination of MB cell migration and metastasis.

We observed moderate survival differences in mice bearing orthotopic xenograft tumors with SMARCD3 deletion or overexpression compared with the controls (Extended Data Fig. 2j). This could be explained by a mechanism whereby SMARCD3 moderately influences tumor cell proliferation, leading to the continuing growth of the primary tumors. However, we grouped these mice to increase cohort size and found a significantly decreased survival in mice with high levels of SMARCD3 expression (MED8A, D458, and D425-SMARCD3 OE) compared with mice with low levels of SMARCD3 expression (MED8A-SMARCD3 KO, D458-SMARCD3 KO, and D425) (Fig. 2n). These data support that SMARCD3-induced metastasis, rather than proliferation, predominantly contributes to a worse prognosis in these mouse models, further supported by the evidence of no correlation between proliferating cell nuclear antigen (PCNA) and SMARCD3 expression in MB patients (Extended Data Fig. 2k). Collectively, \textit{in vitro} and \textit{in vivo} loss/gain-of-function studies aligning with patient data analysis suggest that SMARCD3 acts as the main driver in MB metastatic dissemination.

**SMARCD3 upregulates DAB1-mediated Reelin signaling to promote MB cell migration**

To delineate molecular mechanisms of SMARCD3 promoting MB metastasis, we performed RNAseq on SMARCD3 KO vs WT MED8A cells. Ingenuity pathway analysis (IPA) based on the 44 downregulated and 67 upregulated DEGs (4-fold change; \(P < 0.05\)) showed the most significant enrichment of Reelin signaling in neurons (Fig. 3a and Supplementary Table 2). Reelin plays a critical role in cell migration and positioning throughout the central nervous system by binding to its receptors, the very-low-density lipoprotein receptor (VLDLR) and/or the apolipoprotein E receptor-2 (ApoER2, encoded by LRP8 gene), and promoting downstream activation of Disabled-1 (DAB1) signaling\textsuperscript{21}. Notably, decreased gene expression of the key components of Reelin signaling, such as \textit{RELN}, \textit{VLDLR}, \textit{DAB1}, and \textit{DCC}, was observed in SMARCD3 KO MED8A cells (Fig. 3b).

DAB1 plays an essential role in Reelin signaling activation, which is mediated by phosphorylation of key tyrosine residues (\textit{e.g.}, Y232) when Reelin binds to VLDLR/ApoER\textsuperscript{21,22}. To test our hypothesis that SMARCD3 upregulates the \textit{DAB1} transcription activity, we validated that DAB1 expression was significantly decreased in SMARCD3 KO MED8A and D458 cells but increased in SMARCD3-overexpressed MED8A, D425, and D556 cells (Fig. 3c, d). Integrated analysis of transcriptomic and proteomic data from MB patient samples\textsuperscript{19} revealed that the \textit{DAB1} mRNA expression was strongly correlated with translational and post-translational modifications of DAB1 protein, including phosphorylation on \textit{serine, threonine}, or \textit{tyrosine} (pSTY), particularly Y232 (Extended
Based on analysis of MB patient datasets\textsuperscript{15,19}, \textit{DAB1} mRNA levels were significantly higher in G3 than those in other MB subgroups and normal cerebellum tissues (Fig. 3e), and DAB1 protein levels also tended to be higher in G3 compared with other MB subgroups (Fig. 3f and Extended Data Fig. 3b). Furthermore, we found positive correlations between SMARCD3 and DAB1 in transcriptional, translational, and post-translational levels (Fig. 3g, h, and Extended Data Fig. 3c) using MB patient datasets\textsuperscript{4,19}. Functional validations revealed that DAB1 deletion significantly decreased cell migration in MED8A (Fig. 3i, j). Analysis of a patient dataset\textsuperscript{4} revealed that \textit{DAB1} expression was associated with MB metastasis (Fig. 3k, l). Together, these results suggest that SMARCD3 transcriptionally regulates Reelin-DAB1 signaling to promote cell migration and MB metastasis.

**Spatiotemporal expression patterns of SMARCD3 relate to Reelin-DAB1 signaling in cerebellar development**

Given a positive correlation between SMARCD3 and DAB1, we asked whether this association exists in other human cancers or normal organs. Pan-cancer analyses using The Cancer Genome Atlas (TCGA) datasets revealed that the levels of \textit{SMARCD3} and \textit{DAB1} mRNA expression were not correlated ($R = 0.17$, $P < 2.2\text{e-16}$) (Extended Data Fig. 3d). While both SMARCD3 and DAB1 were highly expressed in low-grade glioma and glioblastoma, their expression levels were not positively correlated in these tumors ($R = -0.11$, $P = 0.0023$) (Extended Data Fig. 3e). Gene expression correlation analysis in various human normal organs revealed that SMARCD3 and DAB1 were significantly correlated and highly expressed in the brain compared with other organs and in the cerebellar hemisphere/cerebellum compared with other parts of the brain, respectively (Extended Data Fig. 3f, g). Analysis of gene-specific patterns of expression variation across organs and species\textsuperscript{23} revealed that SMARCD3 and DAB1 expression varied considerably across organs and little across species (Extended Data Fig. 3h), indicating potential evolutionary conservation of organ-specific gene expression throughout vertebrates. These data suggest that SMARCD3 regulating DAB1-mediated Reelin signaling is unique to the cerebellum in physiological conditions and MB in pathological conditions.

Reelin signaling is known to critically control PC radial migration and cerebellar circuit function in brain development\textsuperscript{13}. Thus, we asked whether SMARCD3 expression is positively correlated with Reelin signaling in the developmental trajectory of the cerebellum. We analyzed scRNAseq data from the developing murine cerebellum\textsuperscript{24}, and found that \textit{Smarcd3}, \textit{Dab1}, \textit{Vldlr}, and \textit{Lrp8} mRNA were highly expressed in PCs (Fig. 4a, b, and Extended Data Fig. 4a). PCs emerge in the ventricular zone (VZ) from embryonic day 10.5 (E10.5) to E13.5 in mice and from gestation week (GW) 7 to GW13 in humans\textsuperscript{25,26} (Extended Data Fig. 4b). Then, PCs migrate toward the outer surface of the cerebellar cortex to subsequently form the Purkinje cell layer (PCL) from E12.5 to the early postnatal days in mice and during GW16-GW28 in humans\textsuperscript{13,27,28}. Reelin secreted by glutamatergic neurons (granule cells, GCs) acts on PCs, and activates its downstream VLDLR/ApoER2-DAB1 signaling pathway to control PC migration\textsuperscript{29,30}. We found low levels of \textit{Smarcd3}, \textit{Dab1}, \textit{Vldlr}, and \textit{Lrp8} but high levels of \textit{Reln} expression in GCs (Fig. 4b). Further analysis of spatial-temporal gene expression revealed a similar trajectory of \textit{Smarcd3} expression and Reelin signaling, particularly \textit{Dab1} expression in PCs; and high levels of \textit{Reln} expression in GCs from E13.5 to the postnatal stages (Fig. 4c and Extended Data Fig. 4c). Moreover, we performed immunofluorescence (IF) staining of SMARCD3 and the PC-specific markers FOXP2 and Calbindin 1 (CALB1), respectively, using mouse cerebellar tissues. Notably, we observed increased levels of SMARCD3 protein expression that colocalize with FOXP2 and CALB1 at E15.5 and postnatal day 0 (P0), respectively; and dramatically decreased levels of SMARCD3 after P0 that remain low or undetectable at P7, P28, and P84 in the mouse cerebellum (Fig. 4d, e).
To validate expression patterns of SMARCD3 and Reelin signaling in the human cerebellum, we analyzed single-nucleus RNA sequencing (snRNAseq) data of 13 human cerebella ranging in age from 9 to 21 post-conceptional weeks\(^{31}\). After defining cell types and assembling cell-type-specific transcriptomes (Extended Data Fig. 4d, e), we found that SMARCD3 was highly expressed and associated with DAB1, VLDLR, and LRP8 expression in PCs; that RELN was exclusively expressed in glutamatergic neurons, including precursor, cerebellar nuclei, and GCs (Fig. 4f). We further analyzed the normalized gene expression data of 291 normal cerebellum samples over four age groups: fetal (year \(\leq 0\)), infants (0 < year \(\leq 3\)), children (3 < year < 18), and adults (\(\geq 18\) years)\(^{15}\). SMARCD3 mRNA expression was increased from \(~GW13\) to \(~GW28\), then dramatically decreased during 1 year postnatal, and maintained at low levels in infant, children, and adult age groups (Fig. 4g, h). Together, transcriptomic analysis of mouse and human developing cerebellum demonstrates that spatiotemporal expression patterns of SMARCD3 are associated with Reelin signaling in controlling PC migration during cerebellar development. Furthermore, GO-term analysis based on the genes that were positively related to SMARCD3 during human cerebellum development revealed enrichment of cell projection assembly and organization, brain development, and response to wounding (Supplementary Table 3 and Extended Data Fig. 4f). Furthermore, gene-disease network analysis revealed enrichment of childhood and adult MB using these SMARCD3-associated developmental genes in DisGeNET (Extended Data Fig. 4g). Collectively, these results indicate that MB hijacks SMARCD3-Reelin-DAB1 mediated cell migration, a neurodevelopmental program in the cerebellum, to promote tumor metastatic dissemination in MB.

**SMARCD3 modulates chromatin accessibility and \(cis\)-transcription elements controlling DAB1 expression in neurodevelopment and MB**

SMARCD3, also known as BAF60C, a subunit of the BRG1/BRM-associated factor complexes, modulates chromatin accessibility and thereby regulates temporal gene expression programs in cardiogenesis\(^{32}\). To determine the functions of SMARCD3 in genome architecture for regulating gene expression involved in cell migration and tumor metastasis, we performed Assay for Transposase-Accessible Chromatin using sequencing (ATACseq) to examine chromatin accessibility genome-wide in SMARCD3 KO vs WT MED8A cells (Extended Data Fig. 5a). Analysis of accessibility using the nucleosome-free fragments (<100 base pairs) and mononucleosome fragments (180-247 base pairs)\(^{33}\) revealed global changes in chromatin accessibility in the absence of SMARCD3 (Fig. 5a and Extended Data Fig. 5b). We found 20,578 ATACseq peaks with increased accessibility and 10,131 peaks with decreased accessibility in SMARCD3 KO vs WT controls out of 144,432 total accessible regions identified (Fig. 5a). Genes (n=725) proximal to these less accessible peaks (positive correlation with SMARCD3) were involved in cellular movement, assembly, and organization by IPA analysis (Fig. 5b). These data suggest that SMARCD3 regulates chromatin remodeling for promoting cell migration and tumor dissemination.

We next assigned these differentially accessible regions to the nearest genes that could be regulated by the \(cis\)-regulatory elements (CREs). Of note, changes of most genes (90.29%) in chromatin accessibility corresponded to changes in gene expression by RNAseq (Fig. 5c). Specifically, the decreased accessibility of DAB1 in the absence of SMARCD3 was consistent with its decreased levels of mRNA expression (Fig. 5c and 3b). To identify the specific CREs in the genome controlling SMARCD3-mediated DAB1 gene regulation, we first defined the topologically associating domain (TAD) regions that were enriched in the DAB1 locus using available Hi-C data\(^{34}\) (Extended Data Fig. 5c). Second, we analyzed ATACseq data between MED8A SMARCD3 KO vs WT and found 4 decreased accessibility regions within the DAB1 locus-containing TAD in the absence of SMARCD3 (Fig. 5d). To explore the functions of these CREs, we performed cleavage under targets and release using nuclease
in SMARCD3 KO vs WT MED8A (Extended Data Fig. 5d). The 4 CREs (CRE1, CRE2, CRE3, and CRE4) were enriched for chromatin accessibility, H3K4me1, H3K4me3, and H3K27ac, which were attenuated in the absence of SMARCD3 (Fig. 5d). Notably, there were obvious changes of CRE2 for accessibility and H3K4me3 at the transcription start site (TSS) of DAB1 between SMARCD3 KO and WT (Fig. 5d), indicating a key function of CRE2 in SMARCD3-mediated DAB1 transcriptional activity.

To validate these CREs involved in DAB1 regulation in cerebellar development and MB, we analyzed a dataset of chromatin immunoprecipitation sequencing (ChIPseq) chromatin modification profiles and RNAseq-based transcriptomics from 5 human G3 MB samples. We first classified the 5 tumors into the SMARCD3 mRNA expression high h, and low l, groups (Extended Data Fig. 5e). Second, the ChIPseq enrichment data from the 4 CREs proximal to the DAB1 locus in each tumor were pooled into H and L groups, respectively. Thus, we observed histone mark enrichment (H3K4me1, H3K4me3, and H3K27ac) at these CREs, particularly CRE2, from the high group compared with the low group (Fig. 5e). We analyzed the ChIPseq datasets from mouse cerebellum and found increased H3K4me3 and H3K27ac signals from E12.5 to P0, but a decreased H3K4me3 and H3K27ac signals at P56, localizing at these CREs of the Dab1 locus, particularly CRE2 (Fig. 5f and Extended Data Fig. 5f), which corresponded to Dab1 expression during mouse cerebellar development (Extended Data Fig. 5g). These data suggest that SMARCD3 epigenetically regulates DAB1 transcriptional activity by controlling chromatin accessibility and histone modifications at cis-regulatory elements in the developing cerebellum and MB.

Spatiotemporal chromatin architecture regulates SMARCD3 transcription in MB and the developmental trajectory of the cerebellum

To examine the epigenetic regulation of SMARCD3 in MB and the cerebellum, we analyzed ATACseq data of MED8A and identified the 7 accessible regions (CRE1-7) proximal to the SMARCD3 locus (Fig. 6a). To define these open chromatin regions as putative CREs regulating SMARCD3 transcriptional activity, we performed CUT&RUN on H3K4me1, H3K4me3, H3K27ac, H3K27me3, and H3K9me3 in MED8A cells and assessed the histone modification abundance at these CREs. Notably, these chromatin regions were enriched with peaks of H3K4me1, H3K4me3, and/or H3K27ac as hallmarks of active or poised enhancers. To verify these CREs involved in SMARCD3 regulation in MB, we analyzed ChIPseq and RNAseq datasets of 5 patient samples and found enrichment of H3K4me1, H3K4me3, and H3K27ac at these CREs in the SMARCD3 expression high h, group compared with the low l, group (Fig. 6b and Extended Data Fig. 5e). Particularly, H3K27ac, a marker of active enhancers and TSS, was significantly enriched at these CREs in G3 compared with other MB subgroups, which corresponded to SMARCD3 expression based on analysis of a previously published RNAseq dataset (Extended Data Fig. 6a, b). To explore the functions of these CREs in mammalian development, we analyzed the temporal expression of the Smarcd3 and the corresponding histone modifications in mouse cerebellum using publicly available datasets. We first analyzed Hi-C data to map the regulatory regions of the mouse Smarcd3 locus in the genome (Fig. 6c). Then, we analyzed the enrichment of histone modifications, H3K4me1, H3K4me3, and H3K27ac, during cerebellar development based on the ChIPseq data. We observed higher enrichment of H3K4me3 and H3K27ac around these CREs in E16.5 and P0 compared with E12.5 and P56, which corresponded to the levels of Smarcd3 mRNA expression at these time points (Fig. 6c, d). These results suggest that the CREs play a crucial role in regulating SMARCD3 transcription through controlling chromatin architecture.
To functionally evaluate the CREs in SMARCD3 regulation, we employed CRISPR/Cas9-mediated in situ genome excision to remove these CREs, leading to transcriptional inactivation of targeted genes (Fig. 6e). qRT-PCR analysis revealed that site-specific excision of CRE1, CRE4, CRE5, CRE6, and CRE7, but not CRE2 and CRE3, resulted in a significant decrease of the SMARCD3 mRNA expression in MED8A cells (Fig. 6f). Of note, two isoforms of the SMARCD3 gene shared the CRE4-7 but not CRE1, indicating divergence in transcriptional regulation whereby we observed decreased SMARCD3 mRNA expression after site-specific excision of CRE4-7 but not CRE1 in D458 cells (Extended Data Fig. 6c). This observation was supported by higher enrichment of H3K4me3 and H3K27ac around CRE1 in MED8A but not in D458 cells (Fig. 6a and Extended Data Fig. 6d). We further found a higher signal of H3K4me3 and H3K27ac enrichment around CRE4-7 regions in metastatic tumor-derived D458 compared with the paired primary tumor-derived D425 cells (Extended Data Fig. 6d), indicating that these CREs are involved in transcriptional activation of the SMARCD3-mediated tumor metastatic dissemination in MB.

To define how these CREs cooperate to regulate SMARCD3 transcription, we analyzed available datasets of the single-cell combinatorial indexing (sci) assay for profiling chromatin accessibility (sci-ATACseq) in the human fetal cerebellum. Analysis of these sci-ATACseq data revealed higher levels of the SMARCD3 expression in the PCs compared with astrocytes, GCs, and inhibitory interneurons, which were concordant with a more open chromatin structure leading to a higher gene activity score by Cicero, an algorithm for quantitative measurement of how changes in chromatin accessibility relate to changes in the expression of nearby genes based on single-cell data (Extended Data Fig. 6e, f). We further found that Cicero links were heavily enriched around the CRE4-7 at the SMARCD3 locus in the PCs compared with the other three cell types (Fig. 6g and Extended Data Fig. 6g). These data suggest that the CRE1-7, particularly CRE4-7, can form chromatin hubs that physically and functionally control SMARCD3 transcriptional regulation.

The chromatin hubs are enriched for physical proximity, interaction with a common set of transcription factors (TFs), and orchestration of histone modifications in gene expression. Therefore, we generated a list of the putative TFs that should meet the following four criteria: 1) they should be differentially expressed in the human fetal cerebellum compared with infants, children, and adults (absolute log2 fold change > 0.5, P < 0.05); 2) they should be positively or negatively correlated to SMARCD3 mRNA expression in the human normal cerebellum (R > 0.25, P < 0.05); 3) they should be positively or negatively correlated to the SMARCD3 mRNA expression in G3 only or all MBs (R > 0.25, P < 0.05); 4) they are defined in the human TF database. CENPA, CSRNP3, EZH2, FOXN3, NFIX, NR2F2, TEF, and ZFHX4 satisfied the above criteria, which were validated by using CRISPR/Cas9-mediated gene deletion in MB cells. qRT-PCR analysis revealed that deletion of EZH2 and NFIX most significantly decreased and increased the SMARCD3 mRNA expression in MED8A cells, respectively (Fig. 6h). Conversely, overexpression of EZH2 significantly increased SMARCD3 mRNA expression in MED8A and D458 cells (Extended Data Fig. 6h). Analysis of transcriptomic data from normal human brain showed that SMARCD3 was positively correlated with EZH2 (R = 0.38, P = 3.1e-06) but negatively correlated with NFIX (R = - 0.33, P = 0.0004) (Extended Data Fig. 6i). EZH2 expression was significantly increased from about 19 GW to 29 GW and then decreased and maintained at a low level in infants, children, and adults (Extended Data Fig. 6j, k); however, the changes of NFIX expression are opposite during cerebellar development (Extended Data Fig. 6l, m). Taken together, these results demonstrate a comprehensive map of a chromatin hub that orchestrates CREs, chromatin accessibility, TFs, and histone modifications in regulating SMARCD3 transcription in the developing cerebellum and MB metastasis (Fig. 6i).

Inhibition of Src kinase activity attenuates SMARCD3-induced metastatic dissemination
We identified an epigenetic program wherein the EZH2/NFIX-SMARCD3-Reelin/DAB1 signaling regulates spatiotemporal developmental trajectories of PCs in the cerebellum, which is hijacked by MB to promote tumor metastatic dissemination. The Reelin-activated Src family tyrosine kinases (SFKs) are required for the phosphorylation of DAB1 that in turn potentiates SFK activation in a positive feedback manner, which plays a central role in the activation of its downstream signaling cascades during cerebellar development. We asked whether SMARCD3 expression levels are elevated in metastatic tumors, leading to activation of SFK and response to SFK inhibitor treatment for clinical application (Fig. 7a). To this end, we assessed the protein levels of SMARCD3 and phosphorylated Src (p-Src) in 10 patient-matched primary and metastatic MBs (Fig. 7b and Supplementary Table 4). IHC analysis revealed a positive correlation between SMARCD3 and p-Src (Y416), both of which were highly elevated in metastatic tumors compared with the paired primary tumors (Fig. 7c-e). To further verify Src activation induced by elevated SMARCD3, we observed that deletion of SMARCD3 reduced the protein levels of p-Src in MED8A and D458 cells and these cell-derived xenograft tumors (Fig. 7f, g, and Extended Data Fig. 7a). Just as SMARCD3 expression patterns, we observed higher levels of p-Src in the tumor margin than in the tumor center (Fig. 2d and 7h).

To test our hypothesis that SFK inhibition can reduce metastatic dissemination, we first examined in vitro attenuation of cell migration at the lower concentration of Dasatinib, an FDA-approved inhibitor of SFKs for leukemia. Transwell assays revealed that 50 nM Dasatinib significantly decreased cell migration of MED8A and D458 cells (Fig. 7i and Extended Data Fig. 7b). Next, Dasatinib was administered orally once daily at the standard dose of 15 mg/kg and a low dose of 7.5 mg/kg for mice bearing D458-derived orthotopic xenograft MB, respectively. BLI and flow cytometry analyses revealed that both standard and low dose Dasatinib decreased spinal metastasis and the percentage of mice carrying CTCs compared with placebo (Fig. 7j, k, and Extended Data Fig. 7c). However, assessment of tumor cell proliferation and apoptosis in these mice revealed that administration with low dose Dasatinib did not significantly decrease the levels of Ki67 and cleaved caspase-3 (Fig. 7l and Extended Data Fig. 7d). The data indicate that inhibition of SFK activity mainly influences cell migration rather than cell proliferation and apoptosis. Together, these results suggest that SFK inhibition may reduce tumor cell migration and metastatic dissemination at a lower and safe dose in MB, indicating a potential repurposing of this drug for the treatment of pediatric brain tumor metastasis in clinical studies.

**Discussion**

The most critical challenge in designing therapies for children with MB is to reduce tumor metastasis. How tumor cells gain motility and migration capacity to detach from the primary site remains largely unknown. In this study, we identified that G3 MB cells hijack a neurodevelopmental epigenetic program to promote metastatic dissemination whereby abnormally elevated SMARCD3 activates the Reelin/DAB1/Src signaling-mediated cell migration. Our findings provide the first evidence that SMARCD3 plays a central role in cerebellar development and G3 MB metastatic dissemination, which sheds light on the development of antimetastatic therapy for MB patients.

Based on unbiased analyses of MB subgroup-specific gene expression, we uncovered higher expression levels of SMARCD3 mRNA and protein in the G3 subgroup, which is strongly associated with tumor metastasis and worse patient prognosis. SMARCD3, a subunit of the SWI/SNF chromatin remodeling complex, regulates gene expression programs that are essential for heart development and function. Under pathological conditions, SMARCD3 was reported to regulate epithelial-mesenchymal transition (EMT) in breast cancer by inducing WNT5A.
signaling. Our previous study demonstrated epigenetic upregulation of WNT5A contributing to glioblastoma invasiveness and recurrence. These previous studies indicate a relationship between SMARCD3 and tumor aggressiveness. However, in this study, we discovered that SMARCD3 epigenetically regulates Reelin/DAB1 signaling that plays a central role in cell migration and positioning throughout cerebellar development. Moreover, we identified a positive correlation between SMARCD3 and DAB1 that is evolutionarily conserved and unique in the cerebellum and MB, supporting our hypothesis that tumor cells hijack developmental signaling to promote tumor progression.

Our data showed that the spatiotemporal expression pattern of SMARCD3 in the developing cerebellum is strongly associated with PC migration. SMARCD3 expression is dramatically decreased at the late stage of PC development when there is no migratory activity after birth in the human and mouse cerebellum, which is regulated by the Reelin/DAB1 signaling pathway. These findings suggest that the SMARCD3-Reelin/DAB1 pathway acts as a modulator in the balance of “Go” and “Stop” signaling in orchestrating cerebellar development. However, SMARCD3-DAB1 signaling is highly activated in MB, leading to tumor metastatic dissemination. We further defined that EZH2 and NFIX regulate SMARCD3 transcriptional activation in opposite ways through a chromatin hub. The roles of EZH2 in MB are controversial and its mechanisms of action are incompletely understood. Previous studies reported that targeting EZH2 has significant antitumor effects in medulloblastoma, including an aggressive G3 MB. Conversely, the inactivation of EZH2 accelerates MB development and progression by upregulating GFI1 and DAB2IP. Besides its histone methyltransferase activity, EZH2 also acts as a transcriptional co-activator in gene regulation involved in aggressive castration-resistant prostate cancer and breast cancer. NFIX, as a member of the nuclear factor I family (including NFIA and NFIB), plays a critical role in regulating granule precursor cell proliferation and differentiation within the postnatal cerebellum. NFIB was reported to repress Ezh2 expression within the neocortex and hippocampus, indicating negative regulation of these TFs in brain development. Our data show that EZH2 and NFIX serve as a core set of TFs for binding to the CREs proximal to the SMARCD3 locus to form a chromatin hub, which controls spatiotemporal gene expression in the cerebellum and MB metastasis. Our findings further suggest that targeting EZH2 for MB therapy is complex and challenging although multiple EZH2 inhibitors are currently active in clinical trials.

This study also provides new perspectives on the development of antimetastatic therapy for MB patients by testing the inhibitory effects of Dasatinib on tumor cell migration and metastatic dissemination. Although good tolerability of Dasatinib was observed in a pediatric phase I trial for patients with leukemia and other solid tumors, another phase I trial study reported that administration of Dasatinib at 50mg/m^2 twice daily resulted in poor tolerance with significant toxicities in combination with crizotinib (an oral c-Met inhibitor) in children with recurrent or progressive high-grade and diffuse intrinsic pontine glioma. Failures in clinical trials for glioblastoma treatment were also observed after administering dasatinib combined with other drugs including erlotinib and bevacizumab. These clinical studies indicate that targeting SFK activation may need more specific context-dependent mechanisms to exert optimal efficacy in brain tumor treatment. In this study, we identified a cerebellum-specific developmental program that spatiotemporally regulates Purkinje cell migration cerebellar development, depending on SMARCD3-DAB1-mediated Src tyrosine kinase activation. MB hijacking this developmental program provides a strong rationale to target its downstream Src activation for reducing tumor metastatic dissemination. We showed that even lower doses of Dasatinib can reach antimetastatic effects, hopefully causing less toxicity in this specific context. Our findings provide a rationale for combining SFK
inhibition, particularly low-lose Dasatinib, with other standard cytotoxic agents in the treatment of patients with G3 MB.

**Methods**

See supplementary materials

**Declarations**

**Acknowledgments**

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**Author Contributions**

Conceptualization, H.Z., B.P., and B.H.; methodology, H.Z., E.E.B, A.C., M.L., S.J.H., and B.H.; investigation, H.Z., B.P., E.E.B, J.Q., B.X., E.A., V.R., V.S., Y.G., Z.L., and W.F.M.; data curation & analysis, H.Z., J.Q., D.A., S.X., F.L., O.S., and Z.H.; resources, S.W., G.X., T.T., A.C.R., S.C.M., E.H.R., C.G.E., D.S., S.A., G.K., S.L., J.N.R., G.K.G., R.M.F., and M.D.T.; writing-original draft, H.Z., and B.H.; writing-review & editing, B.E.S., K.H., A.B., I.F.P., and S.J.H; supervision, X.L., I.F.P., R.M.F., S.J.H., M.D.T., and B.H.

**Declaration of Interests**

The authors declare no competing interests.

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**Figures**
Figure 1

High levels of SMARCD3 expression in G3 relate to MB metastasis. **a,** A heatmap of gene expression in four MB subgroups and normal tissues. 2-fold change; false discovery rate (FDR) < 0.05. **b,** Venn diagram showing the overlapping SMARCD3 between G3-associated genes and epigenetic genes. **c,** Violin plot showing SMARCD3 mRNA expression using MB patient transcriptomics data. **d,** UMAP visualization and violin plot showing SMARCD3 mRNA expression based on scRNAseq from 25 MB patients. **e,** Boxplot showing protein levels of SMARCD3 expression. **f,** Kaplan-Meier survival curve of MB patients by SMARCD3 mRNA expression. **g,** Representative images of IHC staining for SMARCD3 protein levels in the MB tissue microarray. Log-rank test for a survival fraction of MB patients based on SMARCD3 protein level. **h,** Top 10 biological pathways of the
SMARCD3-associated genes in MB by GO analysis (Spearman's rank correlation coefficient > 0.3 and $P$ value < 0.05). i, Density plots and boxplots showing the association between metastasis status (0, no metastasis; 1+, metastasis at diagnosis) and expression levels of \textit{SMARCD3} mRNA and protein in primary MB samples. j, qRT-PCR and immunoblotting (IB) analyses showing SMARCD3 mRNA and protein levels in 6 G3 MB cell lines. k, Representative H&E images showing primary tumors (yellow dash lines) and brain/spinal metastatic tumors (red dash lines) in 6 G3 MB cell line-derived orthotopic xenograft models. $P$ value was calculated by FDR corrected Welch's t test (c, e, i). $\star\star\star\star P < 0.0001$. Each dot represents one MB bulk sample (c, e, i) or one MB cell d. See also Extended Data Fig. 1 and Supplementary Table 1.
**Figure 2**

**SMARCD3 promotes cell migration and tumor metastasis.**

a, IB for SMARCD3 expression in MED8A with control (WT) and SMARCD3 KO by two independent sgRNAs (KO-1 and KO-2). b, Representative images showing cell migration of MED8A with SMARCD3 WT, KO-1, and KO-2 by transwell assay. c, Representative luminescence images of mice bearing MED8A with SMARCD3 WT or KO-1 cells after implantation. d, Representative IHC staining of SMARCD3 in MED8A-derived xenograft MB tumors. High magnification images show a part of the tumor margin and core areas. e, IB for SMARCD3 expression in D458 with SMARCD3 WT and KO-1. f, Representative luminescence images of mice bearing D458 with SMARCD3 WT or KO-1 after implantation. g, Representative bright-field and fluorescence microscopy images of the mouse brain bearing D458 with SMARCD3 WT and KO. h, Flow cytometry analysis of GFP+ CTCs from peripheral blood mononuclear cells (PBMCs) of mice bearing D458 with SMARCD3 WT and KO. i, qRT-PCR and IB for the expression levels of SMARCD3 mRNA and protein in D425 with vector and SMARCD3 OE. j, Representative luminescence images of mice bearing D425 with vector or SMARCD3 OE after implantation. k, Flow cytometry analysis of GFP+ CTCs from PBMCs of mice bearing D425 with vector or SMARCD3 OE. l, Representative bright-field and fluorescence microscopy images of the spinal cords from mice bearing D425 with vector or SMARCD3 OE. m, Representative fluorescence stereoscopic images of mouse brain tumors derived from D425 with vector or SMARCD3 OE. Inside high magnification images are donated; Boxplot showing the number of brain metastasis. n, Kaplan-Meier survival curve of the grouped mice bearing cells with high (MED8A, D458, D425-SMARCD3 OE) and low (MED8A-SMARCD3 KO, D458-SMARCD3 KO, D425) levels of SMARCD3 expression. The red arrow denotes the metastatic tumor by IVIS imaging (c, f, j). Data are presented as mean ± SD (b, i, m). P values were calculated using one-way ANOVA with Dunnett's multiple comparison test (b, n) or a one-tailed unpaired t test (i, m). ****P < 0.0001. See also Extended Data Fig. 2.
Figure 3

**SMARCD3** promotes MB metastasis through the Reelin/DAB1-signaling pathway. **a.** IPA canonical pathway enrichment analysis of the DEGs in MED8A with SMARCD3 KO vs WT. **b.** Volcano plot illustrating the DEGs in MED8A with SMARCD3 KO vs WT (adjusted \( P < 0.05 \); two-fold change). **c.** qRT-PCR analysis of **DAB1** mRNA expression in MED8A and D458 cells with SMARCD3 KO vs WT. **d.** qRT-PCR analysis of **DAB1** mRNA expression in MED8A, D425, and D556 with SMARCD3 OE vs vectors. **e.** Violin plot showing **DAB1** mRNA expression in GBM and normal cerebellum. **f.** Boxplots showing expression levels of the total DAB1 and phospho-DAB1 (Y232) protein in the proteomics datasets. **g.** Scatterplot showing the correlation between **SMARCD3** and **DAB1** mRNA expression in 1,280 MBs. **h.** Scatterplots showing the correlations between **SMARCD3** and total or phospho-DAB1
protein expression in 45 MBs. i, qRT-PCR analysis of DAB1 mRNA expression in MED8A with DAB1 KO (3 independent sgRNAs) vs WT. j, Representative images and quantification of cell migration of MED8A with DAB1 KO vs WT in transwell assays. k, Bar diagrams showing the percentage of MB patients with/without metastasis (0, no metastasis; 1+, metastasis at diagnosis) between high and low DAB1 mRNA expression. l, Boxplot showing DAB1 mRNA expression in MB patients with metastasis vs without metastasis. Each dot represents one patient bulk sample (e-h); data are presented as mean ± SD (c, d, i, j); P values were calculated using a one-tailed unpaired t test (c, d), FDR corrected Welch's t test (e, f), Spearman's rank correlation analysis (g, h), and one-way ANOVA with Dunnett's multiple comparison test (i, j), ****P < 0.0001. See also Extended Data Fig. 3 and Supplementary Table 2.
Figure 4

**SMARCD3 regulates Reelin/DAB1 signaling in the developing cerebellum.** a, UMAP visualization and marker-based annotation of cell types from developing mouse cerebellum. b, Dotplot showing gene expression in indicated cell types from the developing mouse cerebellum. c, The gene mRNA expression in mouse PCs and GCs along with the cerebellar development. d, Boxplot showing fluorescence intensity of SMARCD3 expression in PCs at each timepoint. e, Representative images of SMARCD3 (red) and FOXP2 (white) or CALB1 (white) in mouse cerebellum at each timepoint. Dashed lines outline indicated cerebellar regions. CP, choroid plexus; EGL, external granule layer; VZ, ventricular zone; NTZ, nuclear transitory zone; RL, upper rhombic lip; RP, roof plate; PCC, Purkinje cell plate; PL, Purkinje layer; IGL, internal granule layer; WM, white matter; ML, molecular layer; GL,
granular layer. **f**, Dotplot showing gene expression in indicated cell types from the developing human cerebellum. **g**, Scatterplots showing changes of SMARCD3 mRNA expression of human cerebella along with the developmental process. **h**, Boxplot showing SMARCD3 mRNA expression levels of human cerebella from indicated age groups. Each dot represents one cell (**a, d**) or a patient bulk sample (**g, h**). Dot color reflects average gene expression and dot size represents the percentage of cells expressing the gene (**b, f**). Data are presented as mean ± SD and *P* values were calculated using one-way ANOVA (**d**) or FDR corrected Welch’s *t* test (**h**). See also **Extended Data Fig. 4** and **Supplementary Table 3**.

**Fig. 5**
SMARCD3 regulates DAB1 transcriptional activation through chromatin remodeling in MB and cerebellar development. a, Volcano plot showing the differential accessibility (log$_2$(fold change) in reads per peak) against the FDR (-log$_10$) of MED8A with SMARCD3 KO vs WT. Each dot represents one peak called by MACS3. b, The top 10 of molecular and cellular function enrichment by IPA using the genes associated with reduced chromatin accessibility (FDR < 0.05; 2-fold change) in MED8A with SMARCD3 KO. c, Pearson correlation analysis of the peak accessibility in ATACseq vs the DEGs in RNAseq. d, ATACseq and histone marker binding signals from CUT&RUN in the DAB1 locus using MED8A with SMARCD3 KO vs WT. The 4 CREs are marked by red bars and dashed line boxes in the genome. e, Histone modification signals at the 4 CREs based on analyzing the ChIPseq data from 5 G3 patient samples. f, Histone modification signals at the CRE2 based on analyzing ChIPseq data from mouse cerebellum at indicated time points. See also Extended Data Fig. 5.
Figure 6

TF-mediated chromatin hubs control SMARCD3 transcriptional activation in cerebellar development and MB. a, ATACseq and histone modification signals from CUT&RUN at the SMARCD3 locus in MED8A. The CREs (1-7) are marked with red bars in the genome and light blue. b, Histone modification signals at the SMARCD3 locus based on analyzing ChIPseq data from 5 G3 patient samples. c, Hi-C chromatin interaction map on a region centered in the Smarcd3 locus in mouse cerebellum (P22). Grey dashed lines outline TAD borders. Histone modification signals based on analyzing ChIPseq data of mouse cerebellum samples at indicated time points. Black arrowheads denote the CREs that are homologous to the CREs in MED8A. d, Histogram of Smarcd3 mRNA expression during mouse cerebellar development. e, The schematic showing CRISPR/Cas9-mediated in situ
genome exclusion by using two sgRNAs to excise a regulatory element in the genome, leading to transcriptional inactivation of the gene. 

f, qRT-PCR analysis of *SMARCD3* mRNA expression in MED8A after CRE excision. 

g, Cicero coaccessibility links among SMARCD3 CREs in PCs using sc-ATACseq data from the human cerebellum. The height and color of connections indicate the magnitude of the Cicero coaccessibility score and the number of the connected peaks. 

h, qRT-PCR analysis of *SMARCD3* mRNA expression in MED8A after indicated TF KO. 

i, The schematic diagram shows a critical role of SMARCD3 transcription regulation mediated by chromatin hubs in cerebellar development and MB metastatic dissemination. Data are presented as mean ± SD from at least 2 independent experiments and *P* values were calculated by one-way ANOVA with Dunnett’s multiple comparisons test (f, h). See also Extended Data Fig. 6.
Figure 7

Targeting SMARCD3-DAB1-Src activation attenuates MB metastatic dissemination. a, The schematic diagram shows that SMARCD3 induces PC radial migration and MB metastasis mediated by the Reelin/DAB1-activated SFK loop. b, Preoperative MRI sagittal image showing a patient with an enhancing metastatic tumor located at peritumoral brain edema in the frontal lobe (red dashed line) and complete resection of the primary tumor in cerebellum (yellow dashed line). c, Scatterplots showing the correlation between the IHC intensity of SMARCD3 and p-Src in MB tumors. Spearman's rank correlation analysis. d, Representative images of SMARCD3 and p-Src IHC staining in the paired primary and metastatic MB from patient P09. e, Quantitative analysis of SMARCD3 and p-Src expression intensity in 10 paired primary and metastatic MBs. f, IHC and quantitative analysis of p-Src and...
total Src protein in tumors derived from mice bearing MED8A and D458 cells with SMARCD3 WT vs KO, respectively. **g**, IB for p-Src and total Src in MED8A and D458 cells with SMARCD3 WT vs KO. **h**, Representative IHC images of p-Src in MED8A-derived xenograft MB tumor. High magnification images show the tumor margin and core areas. **i**, Representative images showing cell migration of MED8A and D458 cells treated with DMSO or 50 nM Dasatinib by transwell assays. **j**, Scheme of experiment in which mice bearing MB were gavaged with placebo, low dose, and standard dose Dasatinib. **k**, Flow cytometry analysis of GFP+ CTCs from PBMCs of the treated mice. **l**, IHC quantitative analysis of cleaved Caspase-3 levels in tumors derived from the treated mice. *P* values were calculated using two-tailed, paired *t* test (e), one-tailed unpaired *t* test (f, i), chi-square test (k), and one-way ANOVA with Dunnett's multiple comparison test (l). See also **Extended Data Fig. 7** and **Supplementary Table 4**.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Zouetal.manuscriptsuppl01.23..2021.docx
- ExtendedDataFigures.pdf
- SupplementaryTable1ThegenesareassociatedwithSMARCD3inMB.xlsx
- SupplementaryTable2ThedownregulatedandupregulatedDEGsafterSMARCD3KO.xlsx
- SupplementaryTable3ThegenesarepositivelycorrelatedtoSMARCD3duringhumancerebellumddevelopment.xlsx
- SupplementaryTable4Clinicalinformationofthe10MBpatients.xlsx
- SupplementaryTable5sgRNAsquencestargetinggenesandCREs.xlsx
- SupplementaryTable6PrimersusedinqRTPCR.xlsx
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- SupplementaryTable10Tablecontainingmousemodels.xlsx
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