

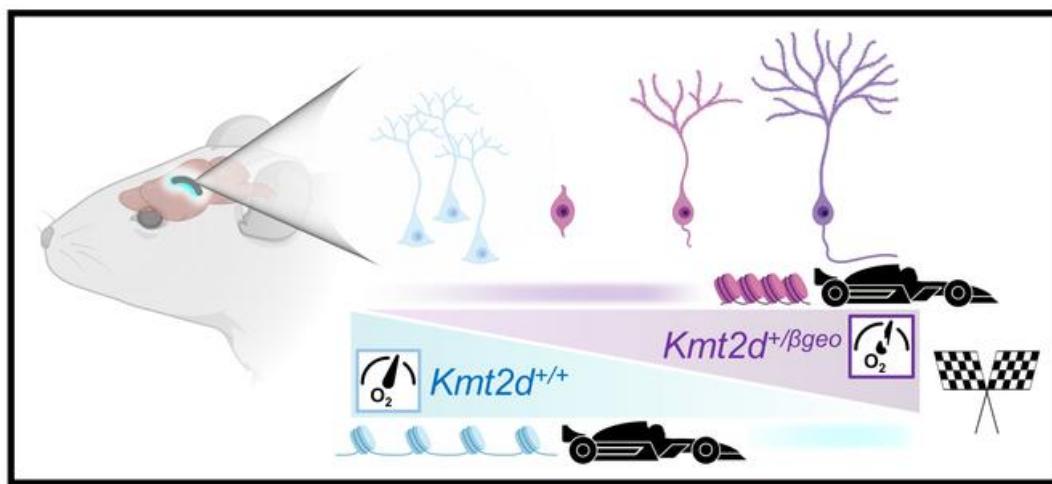
Precocious neuronal differentiation and disrupted oxygen responses in Kabuki syndrome

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JCI Insight. 2019. <https://doi.org/10.1172/jci.insight.129375>.

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Graphical abstract



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1 **Precocious neuronal differentiation and disrupted oxygen responses**
2 **in Kabuki syndrome**

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29 Conflict of interest: The authors declare no conflict of interest.

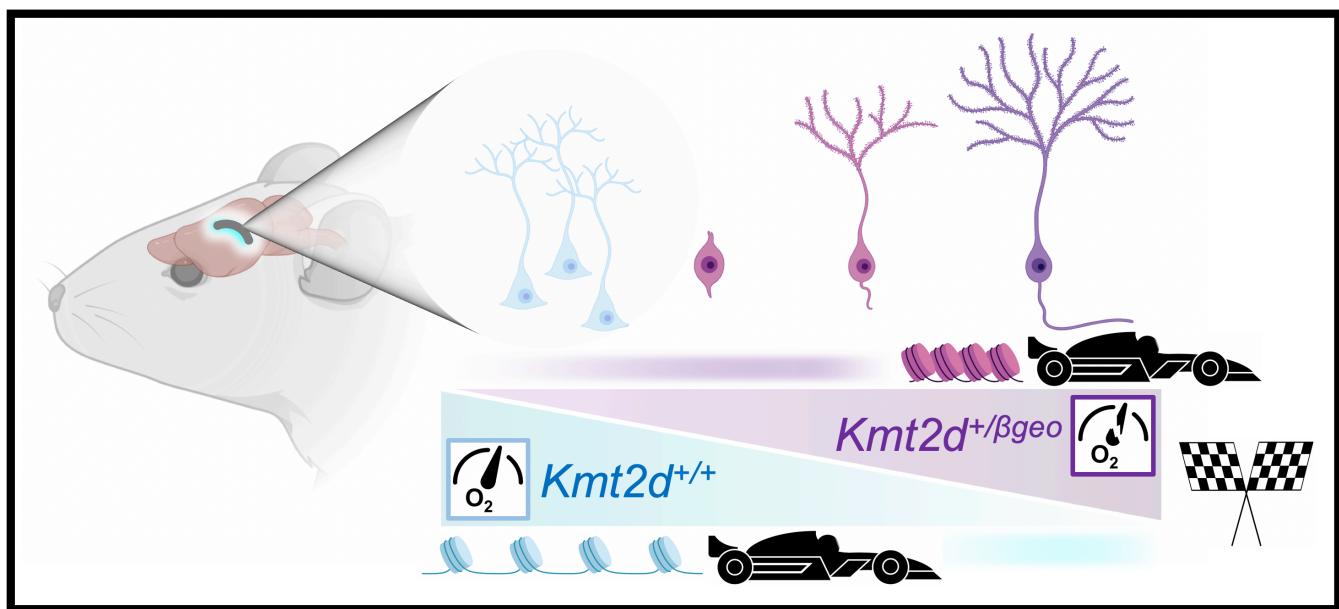
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32 **Abstract**

33 Chromatin modifiers act to coordinate gene expression changes critical to neuronal
34 differentiation from neural stem/progenitor cells (NSPCs). Lysine-specific methyltransferase 2D
35 (*KMT2D*) encodes a histone methyltransferase that promotes transcriptional activation, and is
36 frequently mutated in cancers and in the majority (>70%) of patients diagnosed with the congenital,
37 multisystem intellectual disability (ID) disorder Kabuki syndrome 1 (KS1). Critical roles for KMT2D are
38 established in various non-neuronal tissues, but the effects of KMT2D loss in brain cell development
39 have not been described. We conducted parallel studies of proliferation, differentiation, transcription,
40 and chromatin profiling in KMT2D-deficient human and mouse models to define KMT2D-regulated
41 functions in neurodevelopmental contexts, including adult-born hippocampal NSPCs *in vivo* and *in*
42 *vitro*. We report cell-autonomous defects in proliferation, cell cycle, and survival, accompanied by
43 early NSPC maturation in several KMT2D-deficient model systems. Transcriptional suppression in
44 KMT2D-deficient cells indicated strong perturbation of hypoxia-responsive metabolism pathways.
45 Functional experiments confirmed abnormalities of cellular hypoxia responses in KMT2D-deficient
46 neural cells, and accelerated NSPC maturation *in vivo*. Together, our findings support a model in
47 which loss of KMT2D function suppresses expression of oxygen-responsive gene programs important
48 to neural progenitor maintenance, resulting in precocious neuronal differentiation in a mouse model of
49 KS1.

50

51 **Graphical Abstract**



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53

54 **Introduction**

55 Trithorax group proteins promote chromatin accessibility by exerting antagonistic functions
56 against Polycomb group transcriptional suppressors to activate gene expression (1). Fine-tuning of
57 cell type transitions during neuronal development from NSPCs depends critically on this duality, as
58 evidenced by severe neurodevelopmental defects caused by variants in numerous chromatin-
59 modifying genes (2). Loss-of-function variants in genes encoding two such enzymes, KMT2D and
60 lysine-specific demethylase 6A (KDM6A/UTX) cause the ID disorder KS (KS1 and KS2, respectively) (3,
61 4). Up to 74% (5) of KS cases result from mutations in *KMT2D* (KS1), encoding a major histone H3
62 lysine 4 (H3K4) methyltransferase which catalyzes chromatin-opening modifications at context-
63 specific targets. Developmental requirements for KMT2D in cardiac precursors (6), B cells (7, 8),
64 muscle and adipose (9), and epithelial tissues (10) have been linked, respectively, to *KMT2D*-
65 associated cardiac, immunologic, and oncogenic phenotypes (11), yet the effects of KMT2D
66 deficiency in neurodevelopment are not yet understood.

67 We previously described a mouse model of KS1, *Kmt2d*^{+/βgeo}, demonstrating characteristic
68 features including craniofacial abnormalities and visuospatial memory impairments, associated with
69 decreased adult-born hippocampal NSPCs in the dentate gyrus (DG) (12). Decreased DG grey matter
70 volume was subsequently observed in KS1 patients (13). The continual birth and integration of new
71 neurons makes adult neurogenesis the most potent form of lifelong plasticity in the mammalian brain
72 (14), though recent studies have disagreed on its extent in humans (15–17). During late embryonic
73 stages, a subset of multipotent NSPCs persists in the DG (18), becoming subject to an array of
74 intrinsic and extrinsic factors affecting NSPC maintenance, i.e. self-renewal, proliferation, and
75 neuronal differentiation, throughout adult life. Mounting evidence tightly links metabolic rewiring (19)
76 and hypoxic states in the DG (20, 21) to cell-intrinsic regulation of NSPC maintenance.

77 Here, we find that KMT2D deficiency strongly suppresses metabolic gene expression and
78 leads to reduced proliferation, abnormal hypoxia responses, and precocious neuronal maturation in
79 multiple KS1 model systems. Importantly, these phenotypes were validated *in vivo* in a KS1 mouse
80 model, supporting a role for these abnormalities in the pathogenesis of KS1-associated ID.

81 **Results**82
83 **Genetic ablation of the *Kmt2d* SET methyltransferase domain disrupts proliferation and cell
84 cycle in a cell-autonomous manner**

85 We first selected the HT22 mouse hippocampal neuronal cell line (22) for analysis of KMT2D
86 catalytic function in neuronal context. gDNA sequence encoding the Su(var)3-9, enhancer-of-zeste
87 and trithorax (SET) methyltransferase domain was deleted by CRISPR-Cas9 with an upstream small
88 guide RNA (sgRNA^{up}) in exon 52, and either sgRNA¹ (exon 54) or sgRNA² (intron 54), resulting in
89 deletions of 565 bp (*Kmt2d*^{Δ1}) or 654 bp (*Kmt2d*^{Δ2}), respectively, as verified by Sanger DNA
90 sequencing, in silico translation, and PCR (**Supplementary Figure 1A-B**). Targeted cells were clonally
91 expanded to establish heterozygous (*Kmt2d*^{+/Δ}) and homozygous (*Kmt2d*^{Δ/Δ}) cell lines for comparison
92 against the parental wild-type line (*Kmt2d*^{++/+}). Both biological replicate alleles, *Kmt2d*^{Δ1} and *Kmt2d*^{Δ2},
93 were represented in present studies thus the combined data are denoted hereafter simply as *Kmt2d*^{+/Δ}
94 or *Kmt2d*^{Δ/Δ}. *Kmt2d* mRNA encoded within the targeted region was ~50% decreased in *Kmt2d*^{+/Δ} cells
95 and absent in *Kmt2d*^{Δ/Δ} cells, while *Kmt2d* mRNA from exons upstream of the deletion site was
96 unaffected (**Supplementary Figure 1C**). Immunofluorescence against KMT2D, detecting a peptide
97 sequence upstream of deletions (**Supplementary Figure 1D**), demonstrated distinctly nuclear KMT2D
98 distribution in *Kmt2d*^{++/+} cells but more diffuse distribution in *Kmt2d*^{+/Δ} and *Kmt2d*^{Δ/Δ} cells, while we
99 observed uniformly nuclear expression of a neuronal nuclear marker, RNA binding protein fox-1
100 homolog 3 (RBFOX3), independent of genotype (**Figure 1A**).

101 Proliferation analysis after equal-density plating revealed cell densities ~52% lower in *Kmt2d*^{+/Δ}
102 cells and ~39% lower in *Kmt2d*^{Δ/Δ} cells, compared to wild-type (**Figure 1B**). This defect was supported
103 by dye-based generational tracking, detecting modestly reduced dilution of a fluorescent tracer, i.e.
104 fewer cell divisions, in *Kmt2d*^{+/Δ} and *Kmt2d*^{Δ/Δ} daughter cells compared to wild-type (**Figure 1C**,
105 **Supplementary Figure 1E**), while initial dye uptake in parental cells was genotype-independent. Flow
106 cytometric analysis of cell cycle occupancy, using marker of proliferation Ki-67 (KI67) and a DNA label,
107 revealed that *Kmt2d*^{+/Δ} cells and *Kmt2d*^{Δ/Δ} cells were enriched for S and G₂ phase, compared to wild-
108 type (**Figure 1D, Supplementary Figure 1F**). To characterize temporal dynamics of cell cycle
109 progression, we synchronized cells in G₂/M phase and analyzed DNA content at timepoints after
110 release (**Figure 1E**). Wild-type cells exited G₂/M phase at higher rates than *Kmt2d*^{Δ/Δ} cells, at 3 hours
111 and up to 18 hours after release. Cell death was profiled by flow cytometric detection of caspase-3/7

112 substrate cleavage to distinguish early apoptotic cells. Compared to wild-types, apoptotic cell
113 proportions were greater in both *Kmt2d*^{+/Δ} cells (~287%) and *Kmt2d*^{Δ/Δ} cells (~478%) (**Figure 1F**).

114 To examine proliferation in primary hippocampal progenitors, we isolated NSPCs from micro-
115 dissected DG of *Kmt2d*^{+/βgeo} mice and wild-type littermates. NSPCs exhibited characteristic expression
116 of NSPC marker nestin (NES), with a minority of cells expressing mature neuron marker calbindin
117 (CALB) (**Figure 1G**). Cells were plated at equal density and pulsed with cell division marker 5-ethynyl-
118 2'-deoxyuridine (EdU), then quantified by confocal microscopy. Compared to wild-type, *Kmt2d*^{+/βgeo}
119 NSPCs demonstrated lower proliferation rates as measured by EdU incorporation and cell density
120 (**Figure 1H**).

121 Findings of proliferation defects, G₂/M cell cycle delay, and increased apoptosis in
122 hippocampal cells bearing *Kmt2d* inactivation by SET domain deletion, together with proliferation
123 defects in primary *Kmt2d*^{+/βgeo} hippocampal NSPCs, support a cell-intrinsic role for KMT2D activity in
124 neurodevelopmental contexts.

125

126 Suppressed transcription of KMT2D-regulated hypoxia response genes upon loss of the KMT2D 127 SET methyltransferase domain

128 We performed high-coverage RNA-seq comparing three *Kmt2d*^{Δ/Δ} clones against the parental
129 *Kmt2d*^{+/+} line, each in technical triplicate, followed by differential expression analysis. Libraries
130 clustered robustly by genotype with clear separation of *Kmt2d*^{Δ/Δ} cells from *Kmt2d*^{+/+} by Principal
131 Component Analysis (PCA), yielding 575 significant differentially-expressed genes (DEGs) at a False
132 Discovery Rate (FDR) of 0.05 in *Kmt2d*^{Δ/Δ} cells compared to *Kmt2d*^{+/+} (**Figure 2A, Supplementary**
133 **Figure 2A-B, Supplementary Table 1**). ~76% of DEGs (436 genes) were downregulated in *Kmt2d*^{Δ/Δ}
134 cells, including known KMT2D targets such as Krueppel-like factor 10 (*Klf10*) (12), revealing strong
135 global transcriptional suppression from *Kmt2d* inactivation. Overrepresentation analysis (ORA)
136 revealed significant enrichment of gene networks among *Kmt2d*^{Δ/Δ} down DEGs, including glycolysis
137 and hypoxia-inducible factor 1A (HIF1A) signaling, while *Kmt2d*^{Δ/Δ} upregulated DEGs were enriched in
138 fewer networks (**Figure 2B**).

139 We reasoned that among *Kmt2d*^{Δ/Δ} DEGs, a subset of genes found to also bind KMT2D itself in
140 wild-type cells would more likely represent direct transcriptional consequences of *Kmt2d* inactivation,
141 whereas non-bound DEGs could reflect secondary effects. We performed chromatin
142 immunoprecipitation followed by high-throughput sequencing (ChIP-seq) using a previously validated
143 ChIP-grade KMT2D antibody (9) in *Kmt2d*^{+/+} HT22 cells. We identified 3,756 KMT2D binding peaks

144 significantly enriched over input (**Supplementary Table 2**), of which ~10% occur inside promoters,
145 ~33% (1,235 peaks) occur within 5 kb of a transcription start site (TSS±5kb), and ~25% occur within 2
146 kb (**Supplementary Figure 2C-F**). To account for promoter and enhancer interactions (9, 10, 23), we
147 reasoned that TSS±5kb peaks, compared to more distal peaks, are more likely to reflect KMT2D cis-
148 regulatory functions on proximal genes, so we refer to these as KMT2D-bound genes. The 1,463
149 observed KMT2D-bound genes (**Supplementary Table 3**) were significantly enriched in mRNA 3'UTR
150 binding, rho GTPase signaling, circadian clock, translation, oxidative stress, HIF1A signaling, and
151 other pathways (**Supplementary Figure 2G**).

152 We then intersected KMT2D-bound genes with *Kmt2d*^{Δ/Δ} DEGs to reveal 74 putative direct
153 target genes (**Supplementary Table 3**), of which ~85% (63 genes) were downregulated (**Figure 2C**),
154 including insulin-like growth factor 1 (*Igf1*), and fos-like antigen 2 (*Fosl2*). At least 20 observed KMT2D-
155 bound, *Kmt2d*^{Δ/Δ} DEGs were previously described as KMT2D targets in other tissues (7, 24). KMT2D-
156 bound, *Kmt2d*^{Δ/Δ} down-DEGs were most significantly enriched for pathways including face
157 morphogenesis, glycolysis, hypoxia response, and proliferation, and surprisingly, 29 of these 63 genes
158 are also HIF1A-regulated (25). Although craniofacial features associate with KS1, enrichment of face
159 morphogenesis genes in HT22 cells likely reflects pleiotropic gene functions. KMT2D ChIP-seq peaks
160 on HIF1A-regulated genes clustered at promoters and enhancers, often overlapping CpG islands in
161 genes such as *Fosl2*, with others clustering at alternative TSSs, as in retinoic acid receptor alpha
162 (*Rara*), or in enhancer-like peaks, as in DNA-damage-inducible transcript 4 (*Ddit4*) (**Figure 2D**,
163 **Supplementary Figure 2H**).

164 A large fraction of KMT2D-bound, *Kmt2d*^{Δ/Δ} DEGs control oxygen-responsive metabolism,
165 warranting interrogation of shared KMT2D and HIF1A binding sites. We first intersected KMT2D
166 peaks with HIF1A peaks previously found in embryonic heart (26), finding 423 overlapped regions
167 (**Figure 2E**). Like KMT2D, HIF1A showed ~10% of peaks located inside promoters, but among shared
168 KMT2D/HIF1A-bound peaks this fraction approached ~40%, supporting cooperative regulatory
169 activity (**Supplementary Figure 2I**). We identified 289 TSS±5kb genes, as defined above, for these
170 overlapped KMT2D/HIF1A-bound peaks, including 8 *Kmt2d*^{Δ/Δ} DEGs (**Supplementary Table 3**). To
171 check if KMT2D/HIF1A-bound genes generalize to other tissues we next interrogated independent
172 gene sets having experimentally validated, hypoxia-induced HIF1A binding in the promoter (27). Of 86
173 validated genes, 5 were KMT2D-bound, *Kmt2d*^{Δ/Δ} down-DEGs, 23.3-fold more than expected by
174 chance (Fisher's Exact Test, p=4.74e-6) (**Supplementary Table 3**). Of 81 genes validated in three or

175 more tissues, 3 were KMT2D-bound, *Kmt2d*^{ΔΔ} down-DEGs: *Klf10*, *Rara*, and *Ddit4* (Fisher's Exact
176 Test, p=0.002).

177 Given the prevalence of oxygen response genes among *Kmt2d*^{ΔΔ} down-DEGs and shared
178 KMT2D/HIF1A targets, we hypothesized a positive regulatory role for KMT2D in transcriptional
179 responses to hypoxia in HT22 cells. We subjected *Kmt2d*^{+/+}, *Kmt2d*^{+Δ}, and *Kmt2d*^{ΔΔ} cells to normoxia
180 (21% O₂) or hypoxia (1% O₂), and measured hypoxia-induced gene expression responses. Analysis of
181 canonical HIF1A targets, vascular endothelial growth factor A (*Vegfa*), Bcl2/adenovirus E1B 19-KD
182 protein-interacting protein 3 (*Bnip3*), DNA-damage-inducible transcript 3 (*Ddit3*), and cyclin-dependent
183 kinase inhibitor 1A (*Cdkn1A*), in *Kmt2d*^{+/+} cells revealed robust upregulations upon hypoxic exposure;
184 in contrast, *Kmt2d*^{+Δ} and *Kmt2d*^{ΔΔ} cell lines failed to induce these genes to comparable levels (**Figure**
185 **2F, Supplementary Figure 2J**). In hypoxic conditions, stabilized HIF1A undergoes nuclear
186 translocation, i.e. activation. We therefore quantified nucleus-localized HIF1A fluorescence under
187 normoxia (21% O₂) and hypoxia (1% O₂) (**Supplementary Figure 2K**). Unexpectedly, in normoxia,
188 *Kmt2d*^{ΔΔ} cells exhibited >2-fold greater HIF1A activation than *Kmt2d*^{+/+} cells. Upon hypoxic exposure,
189 HIF1A activation doubled in wild-type cells, but failed to respond in *Kmt2d*^{+Δ} cells and *Kmt2d*^{ΔΔ} cells.

190 Taken together, our data suggest that KMT2D plays an important role in positively regulating
191 HIF1A-inducible, oxygen-responsive metabolic gene programs in neuronal cells.
192

193 **KS1 patient-derived cells recapitulate KMT2D-associated defects in proliferation and cell cycle**

194 We reprogrammed skin biopsy fibroblasts to generate induced pluripotent stem cells (iPSCs)
195 from a previously described female KS1 patient bearing a heterozygous nonsense *KMT2D* mutation
196 (c.7903C>T:p.R2635*) with characteristic facial features, congenital heart disease, and visuospatial
197 memory impairments (28). We selected KS1 iPSCs (KS1-1) bearing normal 46, XX karyotype
198 (**Supplementary Figure 3A**) and characteristic morphology (**Figure 3A**) for comparison against
199 previously described iPSC lines from unrelated healthy controls (C1-2, C3-1) (29). *KMT2D* mRNA
200 quantification in KS1 iPSCs confirmed decreased message compared to controls, as expected due to
201 haploinsufficiency (**Supplementary Figure 3B-C**). Quantification after EdU pulse demonstrated lower
202 proliferation rates (~25%) in KS1 iPSCs compared to controls (**Figure 3B**), accompanied by a shift in
203 cell cycle occupancy (**Figure 3C, Supplementary Figure 3D**) favoring G₂/M phase (24% more cells).

204 We next generated NES-expressing NSPCs through parallel differentiation of KS1 and control
205 iPSCs, using an established small molecule inhibition protocol (30). RT-qPCR confirmed decreased
206 *KMT2D* in KS1 NSPCs (**Supplementary Figure 3E**), and cells displayed normal morphology

207 independent of genotype (**Figure 3D**, **Supplementary Figure 3F**). EdU incorporation rates revealed
208 KS1 NSPCs had a marked proliferation defect (~47% reduced, **Figure 3E**) and fewer mitotic divisions
209 (**Supplementary Figure 3G**). KS1 NSPCs did not display a cell cycle defect (**Figure 3F**,
210 **Supplementary Figure 3H**), suggesting either cell-type dependence or loss of this phenotype during
211 in vitro differentiation. Flow cytometry indicated higher proportions of dying cells in KS1 samples
212 compared to controls among both iPSCs (~130%) and NSPCs (~115%) (**Figure 3G**, **Supplementary**
213 **Figure 3I-J**).

214 To determine whether G₂/M bias, seen in KS1 iPSCs, occurred in unmanipulated primary cells
215 from additional KS1 patients, we analyzed fibroblasts from three molecularly confirmed KS1 patients
216 (KS1-1, KS1-2, KS1-3) and healthy controls. Fibroblasts were synchronized in G₂/M phase followed by
217 flow cytometric analysis of DNA content. At 3 hours post-release, control cells had exited G₂/M phase,
218 in contrast to KS1 cells which remained in G₂/M (**Figure 3H**). Thus, delayed G₂/M exit was consistent
219 in primary, non-reprogrammed cells from three KS1 patients.
220

221 **Transcriptional suppression of metabolic genes in cycling cells, and precocious neuronal**
222 **differentiation in KS1 patient-derived NSPCs**

223 To interrogate transcriptional consequences of *KMT2D* loss in the context of neuronal
224 differentiation, we performed single-cell RNA sequencing (scRNA-seq) in iPSCs and NSPCs from the
225 KS1 patient and controls (**Supplementary Figure 4A**). By inspecting expression of cell-type markers,
226 we confirmed that libraries segregated into clusters reflecting distinct cell identities of the expected
227 lineages (**Supplementary Figure 4B-D**).

228 First, differential expression analysis in iPSCs and NSPCs identified genes downregulated or
229 upregulated in KS1 patient relative to healthy controls (**Supplementary Figure 4E**). KS1 iPSCs
230 displayed strong transcriptional suppression among 421 DEGs, with 372 genes down and 49 genes
231 up (**Supplementary Table 4**). NSPCs showed less directional bias, having 346 significant DEGs
232 among which 147 genes were down and 199 genes were up (**Supplementary Table 5**). Intersection of
233 KS1 iPSC and NSPC DEG lists showed that 40 genes were shared down and 10 genes were shared
234 up (**Supplementary Figure 4F-G**, **Supplementary Table 6**). Shared down genes included glycolysis
235 genes, aldehyde dehydrogenase 7 family member A1 (*ALDH7A1*), enolase 1 (*ENO1*), and
236 triosephosphate isomerase 1 (*TPI1*), as well as factors important to stem cell maintenance including
237 proliferation-associated protein 2G4 (*PA2G4*) and protein lin-28 homolog A (*LIN28A*). As in *Kmt2d*^{ΔΔ}
238 HT22 cells, downregulated genes in KS1 patient iPSCs and NSPCs were significantly enriched for

239 HIF1A direct targets, genes containing the hypoxia-responsive element (HRE) 5'-RCGTG-3' motif, and
240 known hypoxia response genes (**Supplementary Figure 4H**).

241 We next focused on NSPCs from KS1 and controls to interrogate transcriptional effects during
242 neuronal differentiation. We used Uniform Manifold Approximation and Projection (UMAP) to visualize
243 single cells in a manner that displays high-dimensionality data while preserving both local and global
244 relationships (31). Control NSPCs were tightly clustered, indicating similar expression profiles, in
245 contrast to a distinct separation of KS1 cells which gradually lessens in a subset (top) of cells that
246 more closely resemble controls (**Figure 4A**). We then partitioned single-cell libraries into
247 developmentally informative subsets as follows. First, we verified that differences in cell cycle phase
248 composition do not account for KS1-associated differential gene expression in NSPCs
249 (**Supplementary Figure 5A, Supplementary Table 7**). Next, we partitioned cells by stage-specific
250 marker expression to define a differentiation trajectory consisting of early or “cycling” NSPCs,
251 “transitioning” NSPCs, and “differentiating” NSPCs (**Figure 4B**). Cycling cells comprised the majority
252 of NSPCs analyzed and exhibited the greatest KS1-associated expression differences, while
253 expression profiles of transitioning and differentiating NSPCs show gradual convergence of gene
254 expression. We analyzed DEGs exclusively within cycling, transitioning, and differentiating NSPC
255 subsets to determine if particular gene networks drive transcriptional differences in a stage-specific
256 manner (**Figure 4C, Supplementary Table 7**). KS1 DEGs in transitioning NSPCs, and to a lesser
257 extent cycling NSPCs, showed enrichment of genes comprising the Notch signaling pathway including
258 delta-like protein 3 (*DLL3*), protein jagged-1 (*JAG1*), transcription factor HES-5 (*HES5*), and cyclin D1
259 (*CCND1*). Cycling NSPCs had DEGs enriched in glycolysis pathways.

260 Apart from increased rates of KS1 cell death (**Figure 3G**), another possible factor in the
261 observed decrease of proliferative KS1 NSPCs (**Figure 3E**) could be a change in cellular
262 differentiation, such as precocious cell maturation, resulting in depletion of cycling precursors. To
263 explore this by scRNA-seq, we examined markers ranging from immature cells (*PAX6*⁺) to the most
264 differentiated cells (*MAP2*⁺) (**Figure 4D-E**). We further restricted analysis to the transitioning and
265 differentiating, i.e. “maturing” NSPC subset (**Supplementary Figure 5B**), defining a trajectory that
266 enabled parsing of cells into binned deciles of increasing maturation (**Supplementary Figure 5C-H**).
267 Quantification of cell densities revealed strong bias of KS1 NSPCs in the most matured bins relative to
268 controls (**Figure 4F**), i.e. greater representation of mature NSPCs from KS1 than controls. These
269 transcriptional signatures were corroborated experimentally at protein level, finding KS1 NSPCs had

increased MAP2 fluorescence and decreased PAX6 fluorescence relative to control using flow cytometry (**Figure 4G-H**).

Together, these results link transcriptional suppression of metabolic gene pathways to cell-autonomous proliferation defects in *KMT2D*-deficient KS1 patient-derived stem cell models, and scRNA-seq data suggest that precocious differentiation could contribute to KS1-associated neurodevelopmental defects.

In vivo defects of neurogenesis and NSPC differentiation in a *Kmt2d^{+/βgeo}* mouse model of KS1

Finally, we asked whether proliferative defects, transcriptional suppression, and precocious differentiation phenotypes validate in vivo, using an established KS1 mouse model. *Kmt2d^{+/βgeo}* mice, bearing a *Kmt2d* truncating mutation, were previously found to exhibit visuospatial memory impairments and fewer doublecortin (DCX⁺) NSPCs in the DG subgranular zone (SGZ) (12, 32), but NSPC lineage progression in *Kmt2d^{+/βgeo}* mice has not been characterized.

We conducted cell cycle and RNA-seq analysis in *Kmt2d^{+/βgeo}* mice, using an EdU pulse paradigm to label adult-born cells. We sampled micro-dissected DG within 1 cell cycle (16 hours) to capture the full complement of dividing NSPCs (**Figure 5A**), then purified EdU⁺ nuclei by fluorescence-activated cell sorting (FACS) (**Figure 5B**, **Supplementary Figure 6A**). DNA content analysis revealed enrichment of G₂/M phase in *Kmt2d^{+/βgeo}* EdU⁺ DG nuclei (**Figure 5C**). We next profiled transcription by RNA-seq in purified EdU⁺ DG nuclei, yielding 827 DEGs (**Supplementary Figure 6B-C**, **Supplementary Table 8**). The 416 down-regulated genes in *Kmt2d^{+/βgeo}* nuclei were enriched for misfolded protein binding, TCA cycle, proteasome complex, oxygen response, and poly(A) RNA-binding genes. Given the observed downregulation of poly(A) RNA-binding genes, we considered the possibility that improper 3'UTR-mediated mRNA metabolism could lead to accumulation of transcripts influencing NSPC maturation. Indeed, despite little overall bias toward up or downregulation in *Kmt2d^{+/βgeo}* DG nuclei, interrogating positive regulators of neuronal differentiation revealed a marked predominance of pro-neural transcripts upregulated, having only 3 genes down but 14 genes up, including copine-1 (*Cpne1*), focal adhesion kinase 1 (*Ptk2*), ras-related protein RAB11A (*Rab11A*), and retinoblastoma-associated protein 1 (*Rb1*). Interestingly, KS1 patient NSPCs also showed upregulated pro-neural genes such as nuclear receptor subfamily 2, group F, member 1 (*NR2F1*) and pro-neural transcription factor HES-1 (*HES1*), while *Kmt2d^{Δ/Δ}* HT22 cells had upregulated brain-derived neurotrophic factor (*Bdnf*) and neuron-specific microtubule component (*Tubb3/Tuj1*). Such pro-neural gene expression observed across KS1 models raises the possibility that NSPC differentiation rates could be altered in *Kmt2d^{+/βgeo}* mice.

303 To examine NSPC lineage progression in vivo, we analyzed stage-specific cell abundances
304 both at steady-state and after birth-dating of adult-born NSPCs by EdU pulse, comparing *Kmt2d*^{+/βgeo}
305 mice to sex- and age-matched *Kmt2d*^{+/+} littermates (**Figure 5D, Supplementary Figure 6D**).

306 At steady-state, we observed significantly fewer NSPCs in *Kmt2d*^{+/βgeo} mice compared to
307 *Kmt2d*^{+/+} mice at all stages (**Figure 5E**). The cell division marker, minichromosome maintenance
308 complex component 2 (MCM2), distinguished NES⁺ NSPCs in the quiescent (MCM2⁻) or activated
309 (MCM2⁺) state. Importantly, quiescent radial glia-like (qRGL, NES⁺MCM2⁻) NSPCs were ~39% less
310 numerous in *Kmt2d*^{+/βgeo} mice, indicating a baseline paucity in the stem cell pool. Activated RGL
311 (aRGL, NES⁺MCM2⁺) NSPCs were ~43% less numerous, and intermediate progenitor cell (IPC, NES⁻
312 MCM2⁺) NSPCs were ~26% fewer. We confirmed prior observations (12, 32) of fewer neuroblast (NB,
313 DCX⁺) NSPCs, finding a 28% decrease in *Kmt2d*^{+/βgeo} mice. By stratifying analysis along the
314 septotemporal axis of the DG, we observed that aRGL NSPC reductions in *Kmt2d*^{+/βgeo} mice were
315 more pronounced in the septal DG than the temporal region (**Supplementary Figure 6E**), congruous
316 with spatial memory defects (12) localized to the septal DG (33). Because DCX⁺ NSPCs migrate
317 radially during maturation, we compared radial distances of DCX⁺ cell bodies from the SGZ plane and
318 observed increased distances in *Kmt2d*^{+/βgeo} mice (**Supplementary Figure 6F**). Finally, despite
319 diminished NSPC populations in *Kmt2d*^{+/βgeo} mice, we observed no numeric differences among mature
320 neurons (RBFOX3⁺) (**Supplementary Figure 6G**), nor were gross anatomical differences seen by MRI
321 volumetric analysis (**Supplementary Figure 6H, Supplementary Table 9**).

322 From these data, we then calculated a lineage progression index to approximate the expansion
323 potential of each successive neurogenic cell type. Although *Kmt2d*^{+/βgeo} mice showed fewer total
324 NSPCs of each type at steady-state, the lineage progression index at each cell-type transition did not
325 differ significantly (**Supplementary Figure 6I**), suggesting that particular cell-type transition
326 impairments are not responsible for the adult neurogenesis defect. However, we did note substantially
327 higher variance of RGL activation rates in *Kmt2d*^{+/βgeo} mice, suggesting impaired coordination of NSPC
328 mitotic entry (**Supplementary Figure 6J**).

329 Pulse-labeling with marker-based imaging enables precise measurement of birth dates, i.e.
330 mitotic division, of specific cell types. To resolve temporal dynamics of NSPC differentiation in
331 *Kmt2d*^{+/βgeo} and wild-type mice, we pulsed adult mice with EdU for a period of 2 weeks, during which a
332 subset of labeled DG cells is expected to reach a late NSPC (NB) stage, characterized by radial
333 extension of a DCX⁺ neuronal process. In contrast, another subset of pulsed cells, bearing a NES⁺
334 qRGL-like process, represents NSPCs that remain in a stem-like state. Thus, by quantifying EdU-
335 labeled cells exhibiting either a DCX⁺ neuronal process (EdU⁺DCX⁺) or a NES⁺ qRGL-like process

336 (EdU⁺NES⁺) (**Figure 5F, Supplementary Figure 7A**), one can compare relative differentiation status,
337 where a higher proportion of EdU⁺DCX⁺ cells would indicate early or precocious maturation. Indeed,
338 though steady-state cell numbers again confirmed fewer total NES⁺ and DCX⁺ NSPCs in *Kmt2d*^{+/βgeo}
339 mice compared to wild-types, among pulsed cells the *Kmt2d*^{+/βgeo} mice exhibited a significantly greater
340 fraction of EdU⁺DCX⁺ immature neurons (**Figure 5F**). In other words, *Kmt2d*^{+/βgeo} DG NSPCs born
341 within the preceding 2 weeks had achieved a more advanced differentiation state than wild-type cells
342 born in the same window.

343 Together, studies of adult neurogenesis dynamics in *Kmt2d*^{+/βgeo} mice suggest that in vivo
344 neurodevelopmental effects of KMT2D loss recapitulate many phenotypes observed initially in vitro
345 using mouse HT22 cells and KS1 human-derived cells. While comparison of gene expression profiles
346 across these KS1 models revealed few individual genes with shared dysregulation among all models,
347 at network level we observed high enrichment of HIF1A regulatory and RNA metabolism pathways in a
348 comparison of all DEGs in these KS1 models (**Supplementary Figure 8A-D**).
349

350 **Precocious differentiation and reduced hypoxia responses in *Kmt2d*^{+/βgeo} primary hippocampal
351 NSPCs**

352 Cellular oxygen availability has previously been directly linked to maintenance and
353 differentiation of embryonic (33) and adult DG (19) NSPCs. Primary hippocampal NSPCs of *Kmt2d*^{+/βgeo}
354 mice showed increased HIF1A activation compared to wild-type NSPCs, and both genotypes showed
355 increased HIF1A activation upon treatment by HIF1A-stabilizing agent dimethyloxaloylglycine (DMOG)
356 for 12 hours (**Supplementary Figure 9A**). We then subjected NSPCs to a standard in vitro neuronal
357 differentiation protocol, quantifying cell marker expression between 0-8 days (**Supplementary Figure
358 10A-B**). Prior to differentiation (day 0), wild-type NSPCs expressed low levels of a mature DG neuron
359 marker, prospero-related homeobox 1 (PROX1), while *Kmt2d*^{+/βgeo} NSPCs surprisingly showed an
360 increase (**Supplementary Figure 10C**). By measuring expression of a pro-neuronal transcription factor,
361 achaete-scute homolog 1 (ASCL1), we observed a baseline decrease (day 0) in *Kmt2d*^{+/βgeo} NSPCs
362 compared to wild-type (**Supplementary Figure 10D**). In contrast, after 2 days in differentiation
363 conditions, *Kmt2d*^{+/βgeo} NSPCs responded with greater ASCL1 expression compared to wild-types, an
364 effect sustained at 4 and 8 days. DMOG treatment increased ASCL1 levels in both genotypes, though
365 to greater magnitude in wild-type than *Kmt2d*^{+/βgeo} NSPCs. Together, these data are consistent with a
366 link between cellular hypoxia response and neuronal differentiation in hippocampal NSPCs (20).
367

368 **Discussion**

369 The ID disorder KS1 is caused by mutations in the histone methyltransferase *KMT2D*, but
370 mechanistic links to neurodevelopmental and cognitive consequences in patients are not yet clear.
371 KS1 diagnoses are typically made after childbirth, but the inherent reversibility of chromatin
372 modifications raises the possibility that a detailed understanding of KMT2D activity in neuronal cells
373 could identify molecular targets for postnatal interventions in KS1-associated ID.

374 Here, we report that KMT2D-deficient human and mouse neurodevelopment models, in vitro
375 and in vivo, demonstrate similar patterns of transcriptional suppression, proliferative defects, and
376 precocious cellular differentiation. These phenotypes were cell-autonomous in vitro, suggesting that 1)
377 chromatin and gene expression studies in neurogenic cell types could yield disease-relevant KMT2D
378 targets and 2) these cellular models provide platforms for screening of novel therapeutic strategies or
379 targeted manipulations. We performed transcriptomic and KMT2D profiling in these models and
380 observed systematic suppression of hypoxia response pathways, particularly among HIF1A-regulated
381 genes that are also directly KMT2D-bound in neuronal cells. Physically overlapping KMT2D- and
382 HIF1A-bound genomic loci were observed across tissues, ~40% of these at promoters, raising the
383 possibility of shared etiologies in embryonically distinct KS1-affected organ systems. Furthermore,
384 KMT2D-deficient neuronal cells, in contrast to isogenic wild-type cells, were unable to mount
385 characteristic hypoxia-inducible gene activation responses when exposed to low-oxygen conditions,
386 demonstrating oxygen response defects in KS1 models.

387 The implication of hypoxia response defects in KS1 suggests clinical relevance of recent
388 findings in neurodevelopmental regulation. First, the adult hippocampal NSPC niche harbors locally
389 hypoxic, but dynamic, microenvironments and the hypoxic state positively influences NSPC survival
390 (20, 21). Thus, compromised hypoxia responses could render cells particularly vulnerable to changes
391 in oxygen levels as experienced by maturing NSPCs as they migrate from DG SGZ vasculature.
392 Second, NSPC maturation is coupled to a metabolic rewiring from glycolysis in early NSPCs, to
393 oxidative phosphorylation in maturing neurons. Zheng and colleagues (19) recently found this
394 metabolic switch, marked by suppression of glycolytic genes, to be essential for neuronal maturation.
395 In KS1 neural models we observed suppression of hypoxia-responsive glycolytic genes accompanied
396 by upregulation of pro-neuronal differentiation genes, and demonstrated precocious maturation of DG
397 NSPCs by in vivo pulsing of adult *Kmt2d^{+/βgeo}* mice, as well as in vitro differentiation of *Kmt2d^{+/βgeo}*
398 primary DG NSPCs. Future studies could determine whether targeted chromatin opening at hypoxia
399 response loci normalizes differentiation dynamics in KS1 NSPCs.

Analogous findings regarding premature activation of terminal differentiation genes, reduced proliferation, and precocious maturation in KMT2D-depleted keratinocytes were recently linked to disorganized epidermal stratification (10). Furthermore, in KMT2D-deficient cardiomyocytes, loss of H3K4me2 at KMT2D-bound hypoxia response genes associated with cell cycle and proliferative defects in heart development (6). In contrast, KMT2D deletion in B cells conferred proliferative advantage and impaired cell maturation, despite significant up-regulation of differentiation genes (8, 9). Thus, while KMT2D's role in enhancer-mediated gene expression during differentiation is well-established (11), phenotypic manifestations appear cell type- and stage-dependent. We now extend KMT2D-associated phenotypes of transcriptional perturbation, hypoxia response, cell cycle, proliferation, and premature differentiation to neuronal contexts. Phenotypic concordance across tissues of disparate embryonic origin suggests that KMT2D targets important to KS1 phenotypes support basic cellular homeostatic functions related to housekeeping, energy production, and cell cycle progression, rather than genes with purely brain-specific function. Furthermore, we report concordant phenotypes both from nonsense *KMT2D* mutations (patient iPSCs and NSPCs), and mutations limited to the KMT2D SET domain (HT22 cells, *Kmt2d*^{+/βgeo} mice), indicating that loss of either gene dosage or catalytic function of KMT2D can be pathogenic.

Present results indicate that adult hippocampal neurogenesis defects, which we previously found to associate with visuospatial memory defects in *Kmt2d*^{+/βgeo} mice, are observable at all stages examined, including fewer quiescent NSPCs in the DG which could indicate either post-natal depletion or altered niche development in the embryo. Despite having fewer total NSPCs, by pulse-chase experiments we observed the *Kmt2d*^{+/βgeo} NSPC population to achieve a more advanced maturation stage than that of wild-type littermates. Interestingly, adult-born NSPCs wield a disproportionately strong influence on DG circuitry and visuospatial learning during younger, but not older, neuronal maturation stages (34). This stage-dependent coupling of NSPC maturation with cognitive outcomes increases the likelihood that accelerated neuronal differentiation rates could negatively impact visuospatial memory acquisition. Furthermore, multispecies comparisons demonstrate that measured decreases in neurogenesis rates are consistent with accelerated neuronal maturation rates across the lifespan (35).

The apparent paradox of increased HIF1A activation, despite blunted hypoxia-responsive expression in *Kmt2d*^{+/Δ} and *Kmt2d*^{Δ/Δ} neuronal cells raises two possibilities. First, chronic HIF1A activity could result in cellular compensatory efforts to downregulate hypoxia response genes. In this case, heterochromatin environments at HIF1A-binding genes could prevent induction. Alternatively, cellular oxygen sensing could be coupled to gene expression through chromatin states in a HIF1A-

433 independent manner. Independent studies recently discovered direct oxygen sensing by KDM6A/UTX
434 (the H3K27 demethylase lost in KS2 patients) as well as the H3K4/H3K36 demethylase 5A (KDM5A),
435 which controlled chromatin states and cell differentiation in a HIF1A-independent manner (36, 37).
436 These findings link hypoxia-induced histone methylation at H3K4, H3K27, H3K9, and H3K36 directly
437 with control of maturation in multiple cell types, further supporting the notion that KS-associated
438 transcriptional suppression, in the adult DG context, could impact NSPC stage-dependent learning
439 (34) via metabolic dysregulation. Hypoxia-upregulated H3K4me3 peaks (37) were enriched in HIF1A
440 target gene promoters, where we presently observed high overlaps in KMT2D/HIF1A-bound loci.
441 Strikingly, loss of KDM5A, whose activity opposes that of KMT2D at H3K4 sites, caused upregulation
442 of hypoxia-responsive genes (37), i.e. an effect opposite to the present KS1-associated suppression of
443 hypoxia response genes such as *Klf10* and *Bnip3l*. Several histone demethylases, and at least 33
444 chromatin modifiers in total, have been shown to impact hypoxia response genes, 11 of these
445 associating with developmental disorders or cancers, yet KMT2D and other histone
446 methyltransferases had not yet been implicated (38).

447 In summary, our findings suggest that KMT2D deficiency disrupts neurogenesis by negatively
448 impacting NSPC maintenance functions including cell cycle, proliferation, and survival, accompanied
449 by decreased adult NSPC numbers and precocious neuronal differentiation. Chromatin and
450 transcriptome profiling identified KMT2D- and HIF1A-regulated gene programs suppressed across
451 KS1 model systems, implicating previously described roles for hypoxia responses in regulating
452 neuronal differentiation. Indeed, we functionally demonstrate KMT2D-dependent HIF1A activation and
453 target gene induction in KS1 neural models, and diminished response to hypoxic conditions during in
454 vitro neuronal differentiation in *Kmt2d^{+/βgeo}* NSPCs. Together, these findings are consistent with an
455 etiological model for KS1-associated developmental changes in which KMT2D loss transcriptionally
456 suppresses oxygen response programs critical to early NSPC maintenance, favoring precocious
457 cellular differentiation during hippocampal neurogenesis.

458

459 **Methods**

460 Media and reagents are listed (**Supplementary Table 10**).

461

462 **Animals**

463 The *Kmt2d^{+/βgeo}* allele (MII2Gt(RRt024)Byg) was generated by Bay Genomics (University of
464 California) through the random insertion of a gene trap vector. *Kmt2d^{+/βgeo}* mice were fully backcrossed
465 to C57Bl/6J background (JAX) over more than 10 generations. Animals were housed in a 14-hour
466 light/10-hour dark cycle with free access to food and water. Experiments compare age- and sex-
467 matched littermates. Genotyping by PCR has been described (12).

468

469 **Primary hippocampal NSPCs**

470 Female C57Bl/6J mice (JAX) were mated to *Kmt2d^{+/βgeo}* males and sacrificed for embryo
471 harvest at embryonic day 18. Micro-dissected DG from *Kmt2d^{+/βgeo}* and *Kmt2d^{+/+}* littermate embryos
472 was processed for NSPC isolation and in vitro differentiation as described (39).

473

474 **Patient-derived iPSCs, NSPCs, and fibroblasts**

475 Skin biopsy fibroblasts were cultured from molecularly confirmed KS1 patients (KS1-1, KS1-2,
476 KS1-3). KS1-1 fibroblasts were reprogrammed using non-integrating Sendai virus vectors (CytoTune-
477 iPS 2.0). 5 days post-induction, cells were transferred to mouse embryonic fibroblast (MEF) feeder
478 plates in iPSC media. 21 days post-induction, high quality colonies were manually selected for
479 propagation and karyotyping by G-banding. Generation of healthy control lines (C3-1 and C1-2) was
480 previously described (29). Feeder MEFs from E13.5 CF-1 mice were mitotically inactivated by
481 irradiation. iPSCs were enzymatically passaged every 4-8 days using collagenase. NSPCs were
482 induced from iPSCs as previously described (30), briefly, by inhibiting glycogen synthase kinase 3
483 (GSK3), transforming growth factor β (TGF-β), γ-secretase, and Notch signaling pathways using small
484 molecules CHIR99021 (4 μM), SB431542 (3 μM), and Compound E (0.1 μM), in the presence of hLIF
485 (10 ng/ml) and ROCK inhibitor (5 μM) for 7 days. NSPCs were split with Accutase and propagated in
486 neural induction medium on a Matrigel.

487

488 **CRISPR-Cas9 deletions in HT22 cells**

489 HT22 mouse hippocampal cells are commercially available, but were a gift of the Goff
490 Laboratory and maintained in HT22 media. sgRNAs targeting two loci spanning the *Kmt2d* SET
491 domain-encoding region, with cut sites in exon 52 and either exon 54 (*Kmt2d^{Δ1}*) or intron 54 (*Kmt2d^{Δ2}*),

492 were integrated into Cas9 plasmid (pSpCas9BB-2A-puro v2.0 (PX459)) and delivered to cells at 20%
493 confluence using Lipofectamine 2000 according to manufacturer protocol. After puromycin selection,
494 mutant cells were identified by PCR (primers listed) and clonally expanded. Following Sanger
495 sequencing, a subset of clones appearing heterozygous by PCR, but found to bear strand invasion,
496 were removed from analyses.

497

498 **RNA-seq in HT22 cells: library preparation**

499 Cells were plated at equal density and sampled at 60% confluence. Total RNA was isolated
500 from three biological replicates of *Kmt2d*^{ΔΔ} clones and *Kmt2d*^{+/+} parental cells using Direct-Zol RNA
501 MicroPrep, and libraries were constructed in technical triplicate using NEBNext Poly(A) Magnetic
502 Isolation Module and NEBNext UltraII RNA Library Prep Kit for Illumina, with size selection by AMPure
503 XP beads, according to manufacturer protocols. Library quantification and quality checks were done
504 using KAPA Library Quantification Kit for Illumina, High Sensitivity DNA Kit on BioAnalyzer, and Qubit
505 dsDNA HS Assay. Paired end 50 bp reads were obtained for pooled libraries using Illumina HiSeq
506 2500.

507

508 **RNA-seq in HT22 cells: data analysis**

509 We first obtained a fasta file with all mouse cDNA sequences
510 (Mus_musculus.GRCm38.cdna.all.fa.gz) from Ensembl
511 (http://uswest.ensembl.org/Mus_musculus/Info/Index, version 91, downloaded January 2018). Then,
512 sequencing reads were pseudoaligned to this fasta file and transcript abundances were subsequently
513 quantified, using Salmon (40). We then used the tximport R package (41) to convert the transcript
514 abundances into normalized gene-level counts, by setting the “countsFromAbundance” parameter
515 equal to “lengthScaledTPM”. Next, we used the edgeR (42, 43) and limma (44) R packages to log2
516 transform these gene-level counts, and normalize each of the samples with the “voom” function using
517 the effective library size (that is, the product of the library size and the normalization factors, the latter
518 of which we computed with the “calcNormFactors” function provided in edgeR). Subsequently, we
519 estimated the mean-variance relationship, and calculated weights for each observation. In order to
520 account for the correlation between technical replicates of the same clone when performing the
521 differential analysis, we fit a mixed linear model, using the function “duplicateCorrelation” from the
522 statmod R package (45) to block on clone. The differential analysis was then performed using the
523 limma R package. Differentially expressed genes were called with 0.05 as the cutoff for the False
524 Discovery Rate (FDR).

525 When performing the principal component analysis, transcript abundances were first converted
526 into gene-level counts using the tximport R package, with the “countsFromAbundance” parameter
527 equal to “no”. Then, we applied a variance stabilizing transformation to these gene-level counts using
528 the “vst” function from the DESeq2 R package (46), with the parameter “blind” set to “TRUE”, and
529 subsequently estimated the principal components (without scaling the expression matrix) using the
530 1000 most variable genes.

531

532 scRNA-seq: library preparation

533 NSPCs were induced in parallel from each iPSC line (KS1-1, C1-2, C3-1) under identical conditions,
534 and passaged three times before sampling. iPSCs were detached from MEF feeders using
535 collagenase (200 units/ml). iPSCs and NSPCs were dissociated to single-cell suspension using
536 Accutase. Cell counts and viability were analyzed using Countess II. scRNA-seq libraries were created
537 with Chromium Single Cell 3' Library & Gel Bead Kit v2 (10x Genomics) according to manufacturer
538 protocol. Targeted cell recovery for each sample was 5,000 cells. Sufficient cDNA for library
539 construction was achieved using 20 amplification cycles for iPSC libraries and 16 cycles for NSPC
540 libraries. Sample indexing was achieved using 11 PCR cycles for iPSC libraries and 5 cycles for NSPC
541 libraries. scRNA-seq libraries were sequenced using Illumina NextSeq 500.

542

543 scRNA-seq: data analysis

544 Sequencing output was processed through the Cell Ranger 2.1.0 preprocessing pipeline using default
545 parameters with the exception of --expect-cells=5000 for `cellranger count` and --normalize=none for
546 `cellranger aggr`. Reads were quantified against hg19 using the 10x reference genome and
547 transcriptome builds (refdata-cellranger-GRCh38-1.2.0). The aggregated raw count matrix was then
548 used as input for the Monocle2 single-cell RNAseq framework. Differential gene expression analysis
549 was performed on all NSPCs and iPSCs with respect to genotype (KS1 patient vs healthy control) and
550 was performed using the Monocle2 (47) likelihood ratio test (0.1% FDR, Monocle2 LRT, Benjamini-
551 Hochberg corrected) with `num_genes_expressed` added as a nuisance parameter to both the full and
552 reduced models. The directionality of the differential gene test was determined by calculating the
553 mean gene expression across all KS1 patient-derived and healthy control cells respectively, evaluating
554 the relative fold change. High-variance genes were selected as those with a positive residual to the
555 estimated dispersion fit and a mean number of reads per cell ≥ 0.0005 . Cell cycle stage was
556 determined by profiling cell cycle associated genes across all cells and assigning cell cycle state using
557 the R/Bioconductor package scran (48). Dimensionality reduction and visualization was performed via

558 UMAP (31) on the log₁₀(counts + 1) of the high variance genes in the NSPC dataset. The first 10
559 principal components were used as input for UMAP using the default parameters of the R/CRAN
560 package *umap*. Cells were assigned to clusters using Monocle2's implementation of the louvain
561 community detection algorithm. Learned clusters were then aggregated by hand based on marker
562 gene expression into three clusters ("Differentiating", "Transitioning", "Cycling"). Differential gene
563 expression within clusters, and between genotypes was performed as described above. The
564 "Differentiating" cluster was then segregated, and a smooth line was fitted using a linear regression.
565 This line was determined to represent the direction of differentiation by examination of marker genes
566 (**Supplementary Figure 5C-H**). The residuals of this fit were then plotted and deciles were calculated
567 containing equal number of cells along the axis of differentiation. The number of cells in each decile
568 was then counted with respect to genotype.

569

570 **ChIP-seq: library preparation**

571 *Kmt2d*^{+/+} HT22 cells were sampled at 70% confluence and processed for pull-down with ChIP-grade
572 KMT2D antibody (Millipore Sigma) according to ENCODE guidelines. Sonicated, reverse-crosslinked
573 chromatin served as input control. Briefly, ~300 million cells per cell line were crosslinked in 1%
574 formaldehyde, quenched with 0.125 M glycine, and cell lysate supernatants were collected for
575 immediate processing or snap-frozen for storage at -80°C. Nuclei (20 million/sample) were diluted in 1
576 ml RIPA buffer were sonicated using Bioruptor for 6 cycles of 5 minutes (60 seconds on/30 seconds
577 off) in ice-cold water bath. Supernatants containing sheared chromatin were pre-cleared with Protein
578 A Dynabeads and incubated overnight at 4°C with 8 µg KMT2D antibody. ChIP DNA was recovered by
579 Dynabead incubation (overnight at 4°C plus 6 hours at room temperature) before 6 sequential salt
580 washes of increasing stringency, then eluted and reverse crosslinked overnight at 65°C. DNA was
581 purified using DNA Clean and Concentrator (Zymo Research) and quantified using High Sensitivity
582 DNA Kit on BioAnalyzer, and Qubit dsDNA HS Assay. DNA libraries were constructed using NEBNext
583 UltraII DNA Library Prep Kit for Illumina and quantified using KAPA Library Quantification Kit for
584 Illumina. Paired end 75 bp reads were obtained for pooled libraries using Illumina HiSeq 2500.

585

586 **ChIP-seq: data analysis**

587 Sequencing reads were aligned to the mouse reference genome (mm10) using Bowtie2 (49). Then,
588 duplicate reads were removed with the function *MarkDuplicates* from Picard
589 (<http://broadinstitute.github.io/picard/>). Peaks were subsequently called using MACS2 (50), with the
590 "keep-dup" parameter equal to "all". After peak calling, we excluded all peaks that overlapped with

591 blacklisted regions provided by ENCODE (51). As a quality metric, using the resulting list of peaks, we
592 computed the fraction of reads in peaks (frip) with the “featureCounts” function in the Rsubread
593 package (52), with the “requireBothEndsMapped” parameter equal to “TRUE”, and the
594 “countChimericFragments” and “countMultiMappingReads” parameters equal to FALSE. We found
595 frip to be 2.9%, which is within the typically encountered range of values for a point-source factor (53).
596 To identify genes likely to be regulated in *cis* by KMT2D, we first obtained the coordinates of 10kb
597 regions centered around the TSS for each gene, using the “promoters” function from the
598 EnsDb.Mmusculus.v79 R package (54), with the “filter” parameter equal to
599 “TxBiotypeFilter(“protein_coding”), and the “upstream” and “downstream” parameters both equal to
600 5000. Subsequently, we selected those genes whose extended promoter (+/- 5kb from the TSS)
601 overlapped with at least one KMT2D peak, using the “findOverlaps” function in the GenomicRanges R
602 package (55).

603

604 **Purification of EdU⁺ nuclei**

605 Mice were given 150 mg/kg EdU by intraperitoneal injection and sampled after 16 hours. DG was
606 micro-dissected in ice-cold PBS immediately following sacrifice by halothane inhalation. Total nuclei
607 were purified as described (56) with addition of RNase inhibitor to all buffers. Briefly, DG was dounce-
608 homogenized in 1 ml lysis buffer and layered above a sucrose gradient for ultracentrifugation at
609 28,600 RPM for 2 hours at 4°C. Nuclei were resuspended in Click-iT EdU AlexaFluor-488 with RNase
610 inhibitor, and incubated 30 minutes at room temperature. Samples were passed through 40 µm filter,
611 stained with 1 µg/ml DAPI, and kept on ice before sorting. Lysates processed identically from non-
612 EdU-injected mice served as negative controls during sorting with Beckman Coulter MoFlo Cell Sorter.
613 Cell cycle analysis by DNA content was performed with gates discriminating 2N and 4N cells by DAPI
614 fluorescence.

615

616 **RNA-seq: EdU⁺ nuclei**

617 Purified EdU⁺ nuclei from 3 *Kmt2d^{+/βgeo}* and 3 wild-type littermate female mice (500 nuclei pooled per
618 genotype) were sorted into Smart-Seq 2 lysis buffer (2 µL Smart-Seq2 lysis buffer with RNase
619 inhibitor, 1 µL oligo-dT primer, and 1 µL dNTPs), briefly spun by tabletop microcentrifuge, and snap-
620 frozen on dry ice. Nuclei were processed according to a modified Smart-seq2 protocol (57). Briefly,
621 lysates were thawed to 4°C, heated to 72°C for 5 minutes, and immediately placed on ice. Template-
622 switching first-strand cDNA synthesis was performed using a 5'-biotinylated TSO oligo. cDNAs were
623 amplified using 20 cycles of KAPA HiFi PCR and 5'-biotinylated ISPCR primer. Amplified cDNA was

624 cleaned using 1:1 ratio of Ampure XP beads and approximately 250 pg was input to a one-quarter-
625 sized Nextera XT fragmentation reaction. Fragmented fragments were amplified for 12 enrichment
626 cycles and dual indexes were added to each well to uniquely label each library. Concentrations were
627 assessed with Quant-iT PicoGreen dsDNA Reagent (Invitrogen) and samples were diluted to ~2nM
628 and pooled. Pooled libraries were sequenced on the Illumina HiSeq 2500 platform to a target mean
629 depth of ~8 x 105 bp paired-end fragments per cycle. Paired-end reads were aligned to mm10 using
630 HISAT2 (58) with default parameters except: -p 8. Aligned reads from individual samples were
631 quantified against a reference genome (GENCODE vM8) using cuffquant (59). Normalized expression
632 estimates across all samples were obtained using cuffnorm with default parameters (60).

633

634 RT-qPCR

635 Total RNA was isolated by RNeasy Mini and cDNA libraries were constructed with High-Capacity
636 cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer protocols.
637 Experiments were performed in technical triplicate, with biological replicates as indicated. Probes
638 were from Taqman.

639

640 Immunostaining, confocal imaging, and processing

641 Coronal brain sections of 30 µm (every sixth slice) were analyzed in serial order. Briefly, adult brains
642 were PFA-fixed by transcardial perfusion and post-fixed for 12 hours before cryoprotection by 30%
643 sucrose in phosphate buffer. Brains were sectioned by cryostat (Leica), directly mounted to charged
644 slides, and stored at -80°C. Antigen retrieval (DakoCytomation) was performed at 95°C for 20 minutes.
645 Overnight incubation at 4°C in primary antibodies (**Supplementary Table 10**) preceded AlexaFluor-
646 conjugated secondary antibody (1:500). Tiled, z-stacked images were acquired using Zeiss LSM780
647 FCS AxioObserver confocal microscope and Zen software (Zeiss) to encompass entire DG structure.
648 Images were quantified using Imaris (BitPlane) by experimenters blinded to genotype. Cell counts
649 were corrected by DG area multiplied by z-thickness, and expressed as cells/mm³. For pulse-label
650 experiments, mice were injected with 150 mg/kg EdU in saline every 48 hours and sampled as above.
651 DCX⁺ neuroblast distance from SGZ plane was measured in Fiji (NIH). Patient-derived cell imaging
652 utilized EVOS FL Cell Imaging System with analysis in Fiji.

653

654 FACS and analysis

655 Flow cytometry analysis with FACSverse and FACSSuite (BD Biosciences), and sorting by Beckman
656 Coulter MoFlo Cell Sorter with proper gate settings and doublet discrimination (**Supplementary**

657 **Figure 3J, Supplementary Figure 6A).** Runs of 10,000 or more cells were analyzed from technical
658 triplicate culture wells and analyzed in FlowJo v10 (Tree Star Inc). Unstained and secondary-only
659 samples served as control. Cells were sampled after 30-minute pulse of EdU (10 μ M) using Click-iT
660 EdU Flow Cytometry Assay (ThermoFisher Scientific). CellTrace Violet and CellEvent caspase-3/7
661 reagent (ThermoFisher Scientific) were used according to manufacturer protocols. For cycle
662 synchronization, 250 ng/ml nocodazole (Sigma) was applied for 18 hours before release.

663

664 **Magnetic Resonance Imaging (MRI)**

665 3D T2-weighted MRI (9.4T) was performed in PFA-perfused brains of *Kmt2d^{+/βgeo}* (n=3) and *Kmt2d^{+/+}*
666 (n=3) female mice aged 4 months. Atlas-based, volume-corrected analysis was performed in 25 brain
667 regions (DtiStudio).

668

669 **Statistics**

670 For high-throughput experiments, see Methods. For cellular assays, see Figure Legends. Statistical
671 analyses with multiple comparisons correction were done with GraphPad Prism (version 7.0b). Gene
672 set enrichments were determined according to WebGestalt (61), or by Fisher's Exact Test in R version
673 3.5.2 as indicated.

674

675 **Study Approval**

676 All mouse experiments were performed using protocols approved by the Animal Care and Use
677 Committee of Johns Hopkins University School of Medicine and are in accordance with NIH
678 guidelines. Informed consent regarding KS1 patient samples was obtained according to institutional
679 IRB and ISCRO protocols approved by JHU.

680

681 **Author contributions**

682 GAC and HTB conceived the study; GAC and HTB wrote the manuscript; GAC, HNN, GC, JDR, LZ
683 performed experiments; GAC, LB, JA, KDH and LG analyzed data.

684

685 **Acknowledgments**

686 HTB is funded through an Early Independence Award from the National Institutes of Health (NIH,
687 DP5OD017877), the Icelandic Research Fund (195835-051), and the Louma G. Foundation. Imaging
688 was performed with NIH support (S10OD016374). Karyotype facility supported by NICHD (1U54
689 HD079123-01A1). FACS was performed at the Bloomberg School of Public Health. We thank Michael

690 Sherman for assistance with image quantification and Manisha Aggarwal for MRI. Schematics created
691 with Mark Sandusky or Biorender.com. Hongjun Song and Kai Ge provided critical reagents and
692 advice. Hal Dietz and Gregg Semenza provided conceptual guidance.

693

694 **Data availability**

695 High-throughput data are publicly available. RNA-seq and ChIP-seq: GEO #GSE126167. scRNA-seq:
696 GEO #GSE126027. Scripts for scRNA-seq analysis are available at
697 <https://github.com/Jaughst7/Kabuki-Syndrome-scRNA-analysis>.

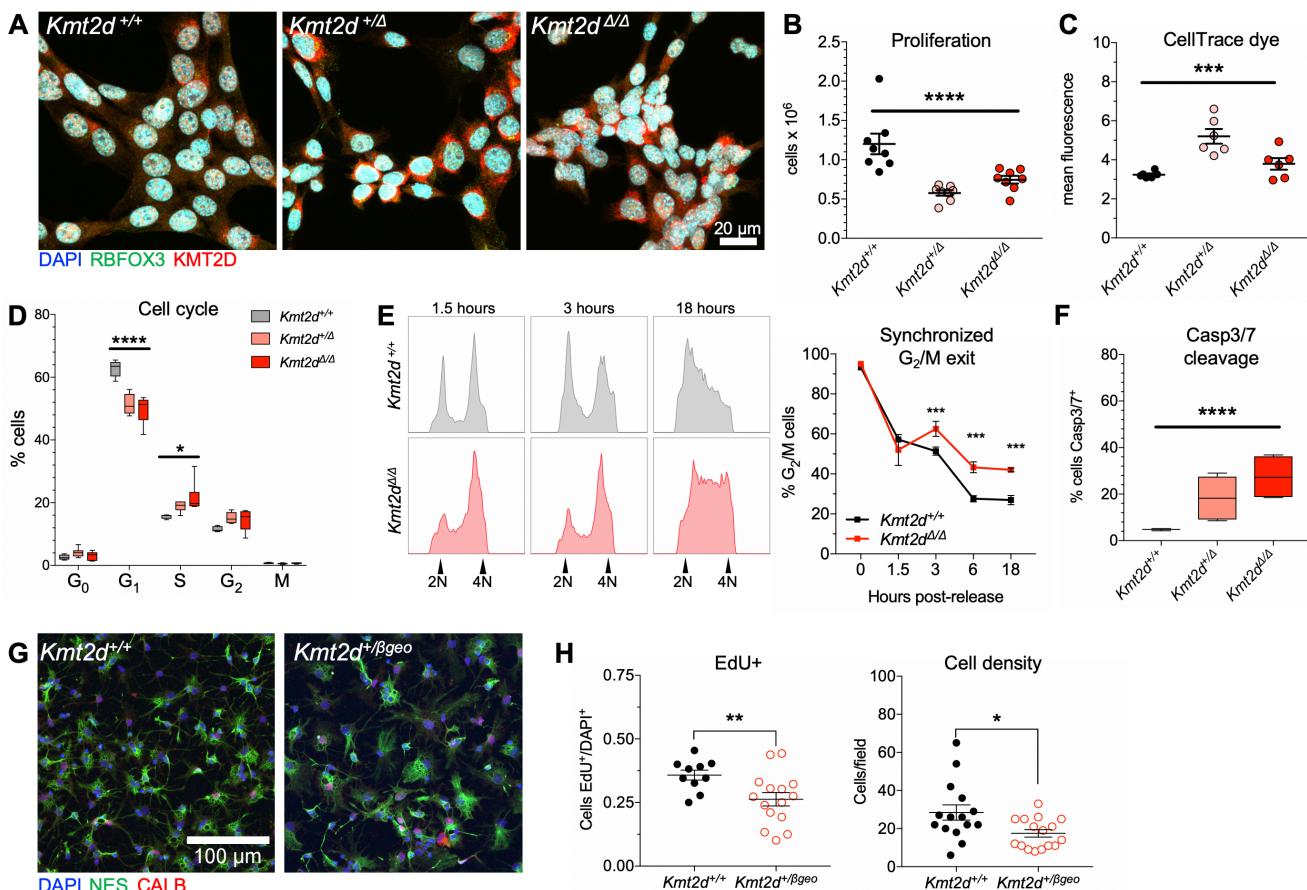
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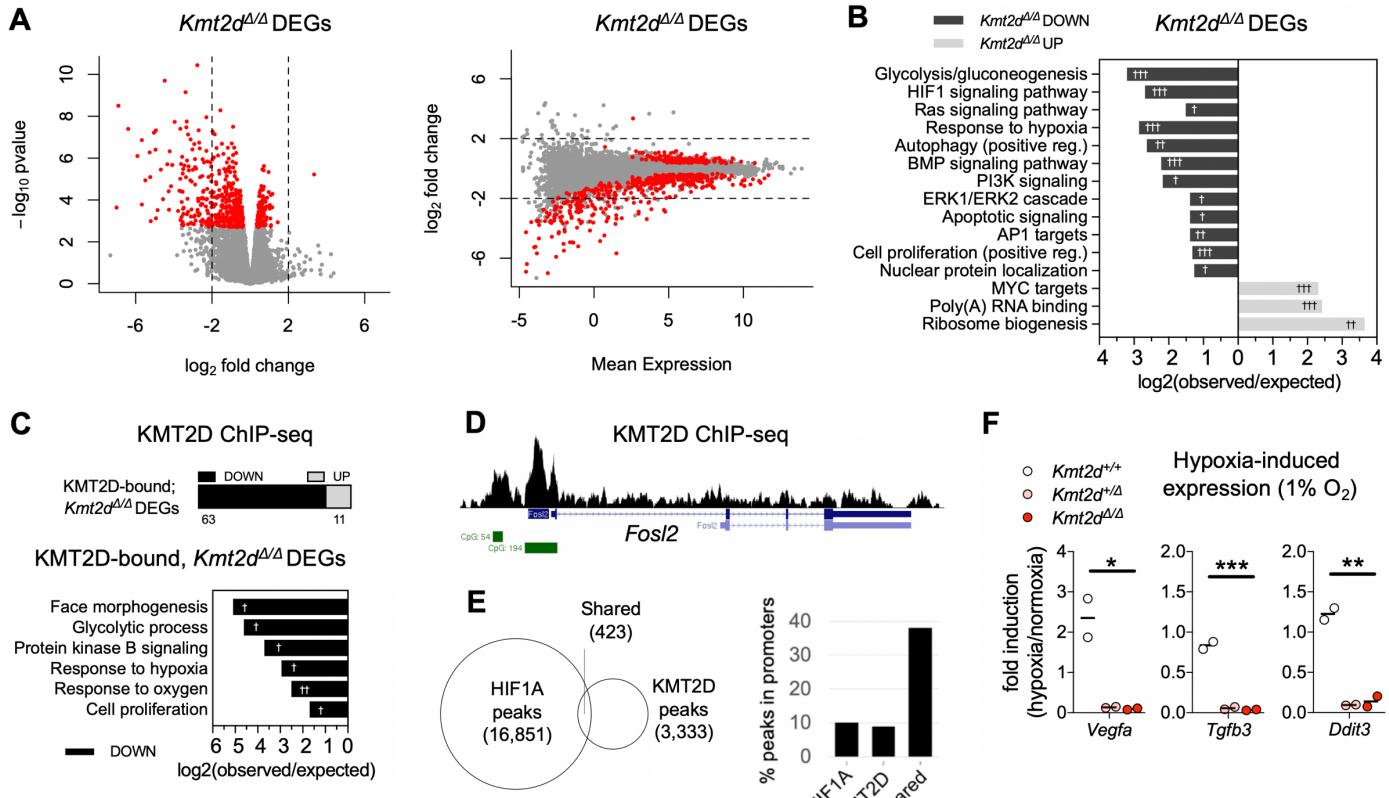
- 828 **Figures**
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Figure 1. Genetic ablation of the *Kmt2d* SET methyltransferase domain disrupts proliferation and cell cycle in a cell-autonomous manner. (A) Representative immunostaining against KMT2D and RBFOX3 in *Kmt2d^{+/+}*, *Kmt2d^{+/\Delta}*, and *Kmt2d^{\Delta/\Delta}* HT22 cells. (B) Decreased proliferation in *Kmt2d*-inactivated cells quantified 72 hours after equal density plating. One-way ANOVA. (C) Generational tracking reveals fewer cell divisions, i.e. reduced dye dilution, of CellTrace Violet in *Kmt2d^{+/\Delta}* and *Kmt2d^{\Delta/\Delta}* cells at 72 hours. One-way ANOVA. (D) Flow cytometric quantification of cell cycle phases using Ki67 and DAPI fluorescence. One-way ANOVA for each cycle phase, independently. (E) *Kmt2d^{+/+}* and *Kmt2d^{\Delta/\Delta}* cells synchronized and released for analysis of G₂/M exit, by DNA content, up to 18 hours after release, and quantification of cells in G₂/M (technical triplicates per time point). Bars indicate mean ± SEM. Two-way ANOVA (P<0.0001) with post hoc multiple comparisons. (F) Flow cytometric quantification of early apoptotic cells by caspase-3/7 fluorescence. One-way ANOVA. (G) Confocal images of nestin (NES) and calbindin (CALB) expressing primary hippocampal NSPCs from *Kmt2d^{+/+}* and *Kmt2d^{+/\beta geo}* mice, and (H) quantified proliferation. Student's t-test. Bars indicate mean ± SEM. Boxes indicate mean ± interquartile range; whiskers indicate minima and maxima. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Scale bars 20 μm (A) or 100 μm (G).



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882 **Figure 2. Suppressed transcription of KMT2D-regulated hypoxia response genes upon loss of**
883 **the Kmt2d SET methyltransferase domain in neuronal cells.** (A) Expression analysis by RNA-seq in
884 HT22 cells reveals 575 significant differentially expressed genes (DEGs) in *Kmt2d*^{Δ/Δ} clones (3 biological
885 replicates) relative to *Kmt2d*^{+/+} cells, each in technical triplicate. Fold changes in expression indicate
886 most significant *Kmt2d*^{Δ/Δ} DEGs (~76%, red dots) are downregulated in *Kmt2d*^{Δ/Δ} cells, plotted against
887 p-value and mean expression. (B) Gene networks significantly enriched among down- or up-regulated
888 *Kmt2d*^{Δ/Δ} DEGs. (C) *Kmt2d*^{Δ/Δ} DEGs which are also KMT2D-bound, as determined by ChIP-seq
889 chromatin profiling in *Kmt2d*^{+/+} HT22 cells, and gene networks significantly enriched among KMT2D-
890 bound, *Kmt2d*^{Δ/Δ} DEGs. (D) Representative ChIP-seq track of a KMT2D-bound, *Kmt2d*^{Δ/Δ} DEG
891 depicting KMT2D binding peaks (black), RefSeq gene annotations (blue), and CpG islands (green). (E)
892 Overlapping loci of observed KMT2D-ChIP peaks in HT22 cells and HIF1A-ChIP peaks in embryonic
893 heart (26). Overlapping KMT2D/HIF1A peak regions, compared to individually bound regions, are
894 enriched at gene promoters. (F) RT-qPCR analysis of hypoxia-induced gene expression in *Kmt2d*^{+/+},
895 *Kmt2d*^{Δ/+}, and *Kmt2d*^{Δ/Δ} cells, following 72 hours in normoxia (21% O₂) or hypoxia (1% O₂), with fold
896 induction of target gene mRNA. 2 biological replicates per genotype, each in technical triplicate. One-
897 way ANOVA. (*p<0.05, **p<0.01, ***p<0.001). Fisher's Exact Test (†FDR<0.05, ††FDR<0.01,
898 †††FDR<0.001).

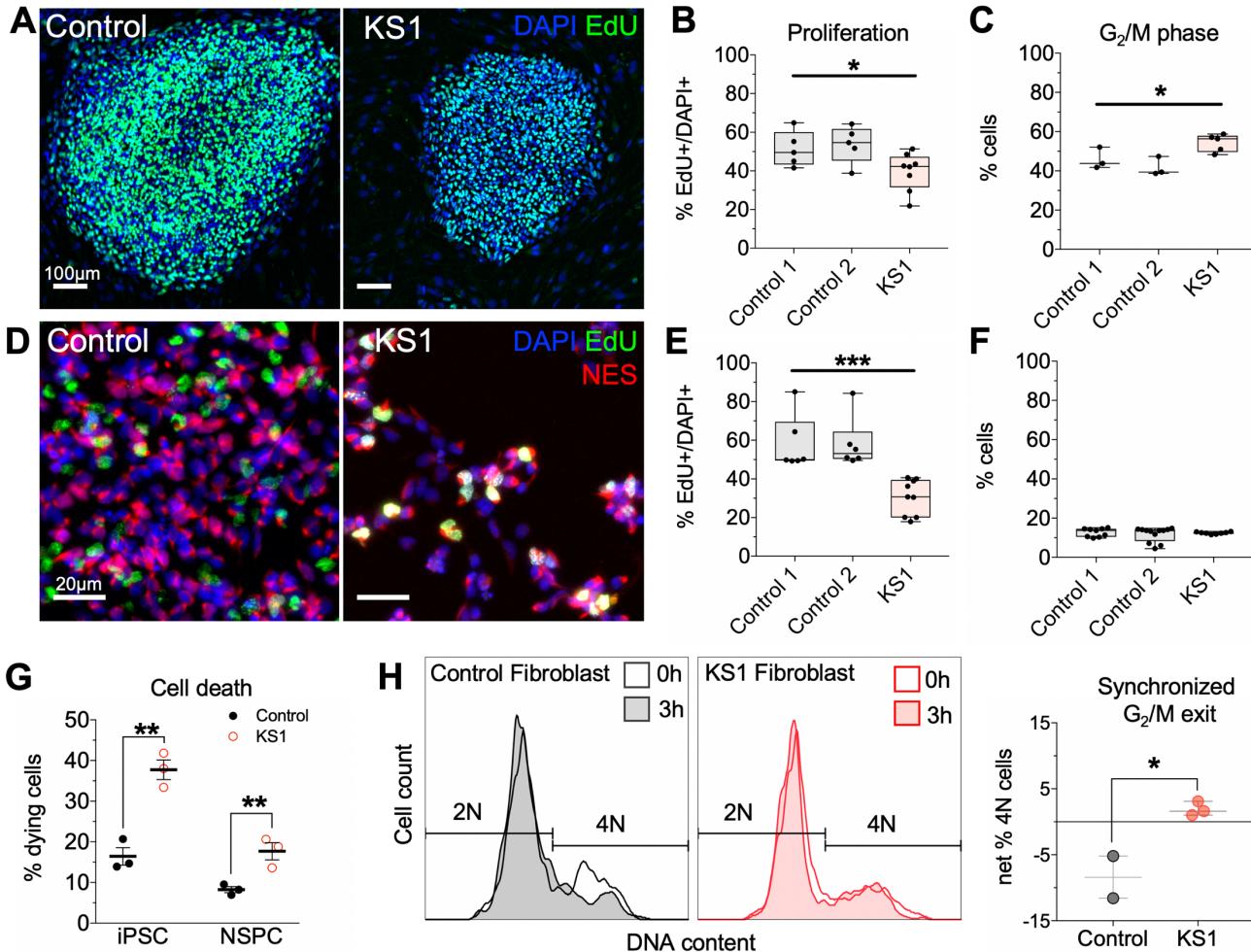
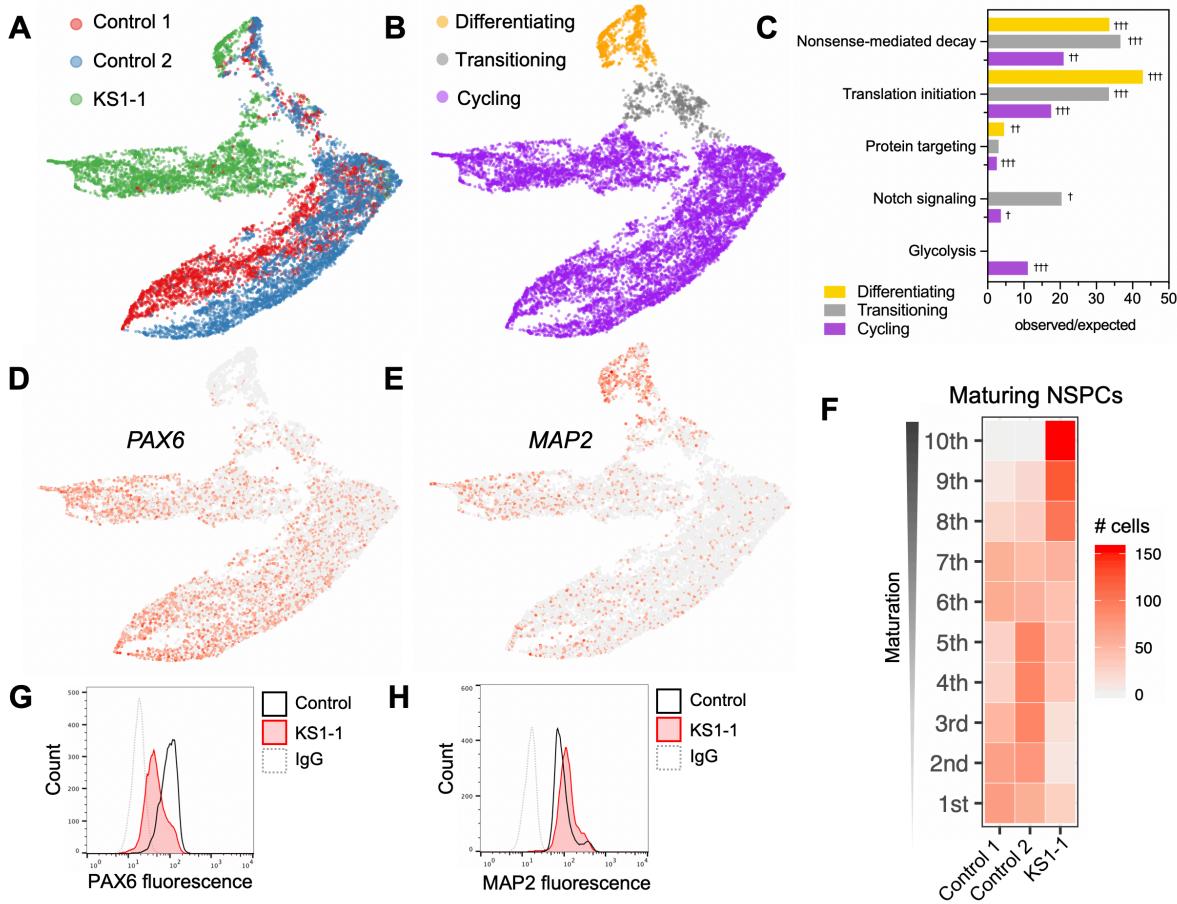


Figure 3. KS1 patient-derived cells recapitulate KMT2D-associated defects in proliferation and cell cycle. (A) Representative immunostaining of iPSCs derived from a *KMT2D*^{+/−} KS1 patient (c.7903C>T:p.R2635*) and healthy controls. (B) Proliferating cells were pulsed with EdU for 30 minutes and quantified by flow cytometry. One-way ANOVA. (C) Cell cycle analysis in iPSCs, discriminating 2N and 4N DNA content (G_1/G_0 and G_2/M , respectively) by flow cytometry using DAPI fluorescence. One-way ANOVA. (D) Representative immunostaining of NES-expressing NSPCs induced from iPSCs of KS1 patient and controls. (E) EdU pulse assay quantified by flow cytometry. One-way ANOVA. (F) Cell cycle analysis in NSPCs. One-way ANOVA. (G) Quantification of dying cells by flow cytometric scatter profiles in KS1 patient and control cells. Student's t-test. (H) Synchronized G_2/M exit analysis by flow cytometry in fibroblasts from KS1 patients (KS1-1, KS1-2, KS1-3) and healthy controls (Controls 3 and 4), in triplicate per cell line. Cells were enriched for G_2/M phase using nocodazole and analyzed by DAPI fluorescence to quantify G_2/M phase cell fractions at 0 and 3 hours after release. Student's t-test. Bars indicate mean ± SEM. Boxes indicate mean ± interquartile range; whiskers indicate minima and maxima. (*p < 0.05, **p < 0.01, ***p < 0.001). Scale bars 100 μm (A) or 20 μm (D).

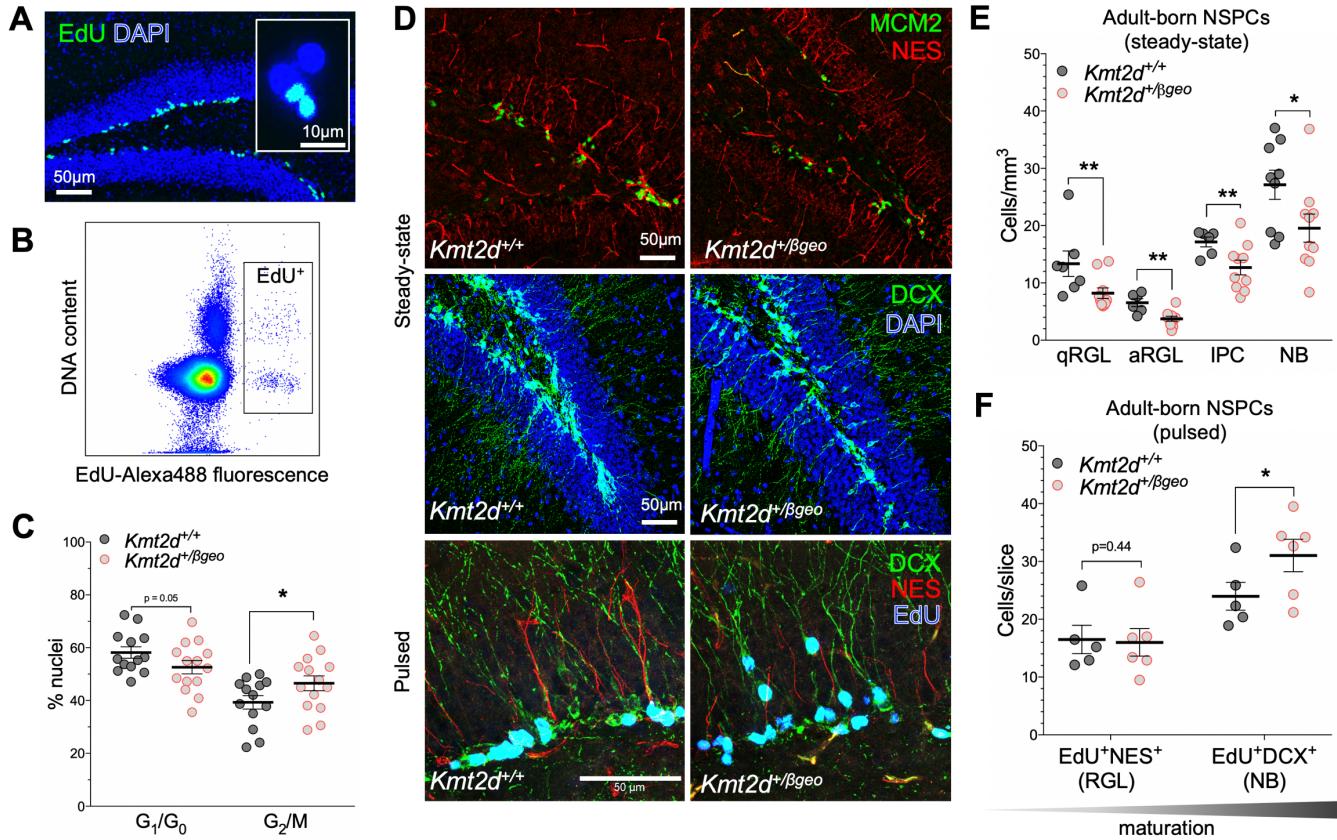
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918 **Figure 4. Transcriptional suppression of metabolic genes in cycling cells, and precocious**
919 **neuronal differentiation in KS1 patient-derived NSPCs.** (A) Single-cell RNA-seq profiling in patient
920 and healthy control iPSC-derived NSPCs (~5,000 cells per patient), with Uniform Manifold
921 Approximation Projection (UMAP) to visualize gene expression differences between cells. (B) NSPCs
922 partitioned by maturation stage as defined by stage-specific marker expression, and (C) enriched gene
923 networks, analyzed exclusively among DEGs for each NSPC subset (cycling, transitioning, and
924 differentiating). (D-E) Representative UMAPs annotated by relative expression intensities of NSPC
925 markers, revealing the maturation trajectory from early NSPCs ($PAX6^+$) to differentiating NSPCs
926 ($MAP2^+$). (F) Heatmap comparing density of NSPCs along the maturation trajectory, defined by binned
927 marker expression from earliest (1st) to most differentiated (10th) deciles, with KS1 cells
928 disproportionately occupying the most mature bins. (G-H) Protein-level experimental validation of
929 marker expression differences by flow cytometry in NSPCs from KS1 patient and controls, plotting
930 fluorescence intensities of PAX6 and MAP2. Fisher's Exact Test (\dagger FDR<0.05, \ddagger FDR<0.01,
931 $+++$ FDR<0.001).

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936 **Figure 5. In vivo defects of neurogenesis and NSPC differentiation in a *Kmt2d*^{+/βgeo} mouse model
937 of KS1**

938 (A) Immunostaining images of dividing (EdU-pulsed) dentate gyrus (DG) NSPCs, and nuclei purified
939 from micro-dissected DG by fluorescence-activated cell sorting (FACS) (B) of labeled nuclei. (C) Cell
940 cycle analysis in purified EdU⁺ DG nuclei from *Kmt2d*^{+/+} and *Kmt2d*^{+/βgeo} mice sampled 16 hours post-
941 pulse, using DAPI fluorescence (13-14 mice per genotype, 200-500 nuclei per mouse). (D)
942 Representative confocal immunostaining of neurogenesis markers in the DG of adult *Kmt2d*^{+/+} and
943 *Kmt2d*^{+/βgeo} mice at steady-state (6-10 mice per genotype, 7-10 z-stack images per mouse) or after
944 EdU pulse (5-6 mice per genotype, 10 z-stack images per mouse). NES⁺ radial glia-like (RGL) NSPCs,
945 in either quiescent (MCM2⁻) or activated (MCM2⁺) states (qRGL and aRGL, respectively), MCM2⁺NES⁻
946 intermediate progenitor cells (IPCs), and DCX⁺ neuroblasts (NB) were quantified. (E-F) Quantification of
947 stage-specific NSPC densities (qRGL, aRGL, IPC, and NB) in adult *Kmt2d*^{+/+} and *Kmt2d*^{+/βgeo} mice at
948 steady-state (E) or after EdU pulse-chase (2 weeks) to birthdate differentiating NSPCs (F). Bars
949 indicate mean ± SEM. Student's t-test (*p<0.05, **p<0.01, ***p<0.001). Scale bars 50 μm, unless
950 specified (A, inset, 10 μm).