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The histone demethylase Kdm6b regulates a mature gene expression program in differentiating cerebellar granule neurons

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Abstract

The histone H3 lysine 27 (H3K27) demethylase Kdm6b (Jmjd3) can promote cellular differentiation, however its physiological functions in neurons remain to be fully determined. We studied the expression and function of Kdm6b in differentiating granule neurons of the developing postnatal mouse cerebellum. At postnatal day 7, *Kdm6b* is expressed throughout the layers of the developing cerebellar cortex, but its expression is upregulated in newborn cerebellar granule neurons (CGNs). *Atoh1*-Cre mediated conditional knockout of Kdm6b in CGN precursors either alone or in combination with Kdm6a did not disturb the gross morphological development of the cerebellum. Furthermore, RNAi-mediated knockdown of *Kdm6b* in cultured CGN precursors did not alter the induced expression of early neuronal marker genes upon cell cycle exit. By contrast, knockdown of *Kdm6b* significantly impaired the induction of a mature neuronal gene expression program, which includes gene products required for functional synapse maturation. Loss of *Kdm6b* also impaired the ability of Brain-Derived Neurotrophic Factor (BDNF) to induce expression of *Grin2c* and *Tiam1* in maturing CGNs. Taken together, these data reveal a previously unknown role for Kdm6b in the postmitotic stages of CGN maturation and suggest that Kdm6b may work, at least in part, by a transcriptional mechanism that promotes gene sensitivity to regulation by BDNF.

Keywords

Cerebellum; Neuronal Differentiation; Histone Demethylase; Kdm6b; BDNF

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Introduction

Neuronal differentiation is comprised of sequential steps that include progenitor proliferation, exit from the cell cycle, migration, synapse formation, and functional maturation. Chromatin regulators play a key role in these processes by dynamically remodeling the chromatin landscape to orchestrate the temporal regulation of gene expression programs. The developing mouse cerebellum serves as an ideal model to identify and examine genetic programs that mediate neuronal differentiation (Wang and Zoghbi, 2001). The cerebellum is particularly useful in chromatin studies because of its utility for studying fate determination and postmitotic maturation of a single predominant neuronal cell-type over the full time course of differentiation (Frank et al., 2015).

Cerebellar granule neurons (CGNs)¹ comprise >99% of cerebellar neurons and >85% of all cerebellar cells (Altman and Bayer, 1997). These cells are derived during the first two postnatal weeks from committed granule neuron precursors (GNPs) that proliferate in the outer portion of the external granular layer (EGL) of the developing cerebellar cortex. Following exit from the cell cycle, GNPs first differentiate into immature CGNs in the inner layer of the EGL then migrate past the Purkinje cells to the inner granular layer (IGL), where they mature and form synaptic connections. We have demonstrated that changes in chromatin accessibility, and the consequent regulation of transcription factor binding at distal enhancer elements, controls the developmentally-regulated programs of gene expression that accompany these stages of CGN differentiation (Frank et al., 2015). The key chromatin regulatory factors that coordinate these steps of CGN differentiation remain to be identified.

In addition to changes in chromatin accessibility, the functional state of enhancer and promoter elements are regulated by the deposition of specific combinations of histone marks (Ernst et al., 2011). Histone methylation is a particularly complex modification that can be associated with either the activation or the repression of gene transcription depending on the specific histone residue that is modified (Black et al., 2012). Methylation of histone H3 at lysine 4 is generally associated with transcriptional activity, with trimethylation (H3K4me3) marking active promoters and monomethylation (H3K4me1) marking transcriptional enhancers. Conversely, trimethylation of histone H3 at lysine 27 (H3K27me3) is associated with gene repression. During cell fate determination, a subset of regulatory elements that control cell-type specific genes is marked by both activating and repressive sites of histone methylation (Bernstein et al., 2006; Mohn et al., 2008). These elements are thought to be “poised” such that the corresponding genes can be rapidly turned on or off during subsequent stages of fate determination by the selective loss of either the activating or the repressive mark. Whether such processes play a role in the postmitotic timing of gene expression in a fate-committed neuron remains unknown.

Steady-state levels of H3K27me3 are established by the functional balance between the enzymes that add this modification (the histone methyltransferases Ezh1 and Ezh2) and the

¹Abbreviations: cerebellar granule neurons (CGNs), granule neuron precursors (GNPs), external granule layer (EGL), internal granule layer (IGL), histone H3 lysine 4 monomethylation or trimethylation (H3K4me1 or H3K4me3), histone H3 lysine 27 trimethylation (H3K27me3), jumonji C domain (JmjC), conditional knockout (cKO), postnatal day 7 (P7), days in vitro (DIV).

enzymes that remove it (Margueron and Reinberg, 2011). Within the Jumonji C (JmjC) domain-containing histone demethylase family, there are two selective H3K27me3 demethylases named Kdm6a (UTX) and Kdm6b (Jmjd3) (Agger et al., 2007; Hong et al., 2007; Hubner and Spector, 2010; Lan et al., 2007). *Kdm6a* is on the X chromosome and escapes X inactivation (Greenfield et al., 1998); its Y chromosome homolog, called *Uty*, lacks H3K27 demethylase activity (Hong et al., 2007; Lan et al., 2007; Shpargel et al., 2012).

Several studies have implicated Kdm6b in multiple aspects of neuronal differentiation and function. Consistent with the evidence from other cell lineages that Kdm6b promotes cellular differentiation (Manna et al., 2015; Pan et al., 2015), loss of *Kdm6b* has been observed to impair neurogenesis in embryonic stem cells (Burgold et al., 2008) as well as neural stem cells in culture (Jepsen et al., 2007) and *in vivo* (Park et al., 2014). Furthermore, constitutive *Kdm6b* knockout mice die at birth of respiratory failure that appears to be due to impaired maturation and function of respiratory circuits in the brain (Burgold et al., 2012), suggesting that Kdm6b may function in the maturation of postmitotic neurons as well. Indeed, we have previously found that Kdm6b is a neural activity-regulated gene product that functions in differentiated hippocampal neurons to promote cell survival (Wijayatunge et al., 2014). However, the functions of Kdm6b in postmitotic stages of neuronal differentiation have not yet been established.

To fill this gap in knowledge, we generated GNP-conditional *Kdm6b* knockout mice and examined the expression and function of Kdm6b in developing CGNs of the mouse cerebellum. We find that *Kdm6b* expression is induced when CGN precursors exit the cell cycle and that Kdm6b is required in CGNs for developmental induction of a gene expression program that mediates mature CGN functions. These data implicate Kdm6b as a regulator of neuronal maturation in addition to its functions at early stages of neuronal differentiation.

Methods and Materials

Mice

We performed all procedures under an approved protocol from the Duke University Institutional Animal Care and Use Committee. LoxP-conditional *Kdm6a* and *Kdm6b* mice were described in (Shpargel et al., 2012) and (Shpargel et al., 2014) respectively. Briefly, the conditional *Kdm6a* strain has loxP sites flanking exon 3 of the 29-exon *Kdm6a* gene on the X chromosome, and the conditional *Kdm6b* strain has loxP sites flanking exons 14–20 (which encode the enzymatic JmjC domain) of the 23-exon *Kdm6b* gene on chromosome 11. A frame-shift induced stop codon is introduced by splicing of exons 13 and 21. *Atoh1*-cre mice (Yang et al., 2008) were purchased from The Jackson Laboratory (Stock #011104), and C57BL6/J mice were purchased from Charles River Labs. Mice were genotyped by tail clipping at weaning and again at the time of tissue harvesting. The *Gt(ROSA)^{26tm1Sor}* LacZ reporter strain (Soriano, 1999) was purchased from The Jackson Laboratory (Stock # 003474). We used both male and female C57BL6/J mice for the experiments in this study.

In situ hybridization

Brains were harvested, flash-frozen in an isopentane/dry ice bath (Thermo Fisher Scientific, Carlsbad, CA) and embedded in OCT (Tissue-Tek). 20 µm coronal sections were cut on a cryostat and the slices were mounted on Super Frost Plus slides (need info). We used three different *in situ* hybridization methods in the course of this study. For screening *in situ* on sagittal sections at P7, digoxigenin (DIG)-labeled riboprobes targeting mouse *Kdm6b* exon III in the antisense direction or the sense controls were primed as follows: *Kdm6b* sense probe 5'-GTCGACCATCGGGCAGTGACCCTC-3'; *Kdm6b* antisense probe 5'-GGATCCGACCTTGGCTCTGTGCTGAC-3'. Hybridized riboprobes were visualized by immunological detection with alkaline phosphatase (AP)-conjugated anti-DIG antibodies (Roche) and developed using 5-brom-4-chloroindolylphosphate/nitroblue tetrazolium (BCIP/NBT; Roche). Images were captured on a Leica DMI4000 inverted fluorescence microscope at 10X magnification and stitched using the Fiji plugin, Stitching (Grid Stitching: Grid Snake by Rows). For fluorescent *in situ* hybridization combined with immunofluorescence, digoxigenin (DIG)-labeled antisense riboprobes targeting mouse *Kdm6b* exon III were used. Hybridized riboprobes were visualized by detection with peroxidase-conjugated anti-DIG Fab fragments (Roche, Indianapolis, IN) and developed using TSA (Perkin Elmer, Waltham, MA). For immunofluorescence, *in situ* slides were blocked in 14% NGS and permeabilized in 0.3% Triton X-100 prior to incubation with mouse α-human Ki67 (purified clone B56, 1:100, BD Bioscience, San Jose CA) followed by Cy2-labeled anti-mouse at 1:500 (Jackson ImmunoResearch, West Grove, PA). For high-resolution quantitative fluorescent *in situ* hybridization of *Kdm6a* and *Kdm6b* in control and *Atoh1*-Cre conditional knockout mice, we used the following RNAscope probes from Advanced Cell Diagnostics (Carlsbad, CA): *Kdm6b* (Cat. #477971-C3), targeted to base pairs 19-1133; *Kdm6a* (Cat #456961-C1) targeted to base pairs 1761-2712; *Pvalb* (Cat #421931-C3); and for analysis in the cKO mice, we made a custom probe raised against base pairs 4350-4950 (Cat #501231-C1. We incubated sections for 30s with Hoechst to label nuclei of all cells for identification of anatomical landmarks, and Z-stack images were taken at 40X on a Leica SP8 confocal microscope and analyzed in Fiji.

Light microscopy and image analysis

P7 brains were sectioned and processed for Hoechst nuclear staining. Images were captured on a Leica DMI4000 inverted fluorescence microscope at 10X magnification and stitched using the Fiji plugin, Stitching (Grid Stitching: Grid Snake by Rows). Immunostaining for *Kdm6a* was performed on fresh frozen sections that were fixed in 4% paraformaldehyde and permeabilized with 1% sodium dodecyl sulfate (Cat #L4509, Sigma Aldrich) for 5 mins prior to blocking in 0.3% Triton X-100 and 10% NGS in 1X PBS. Slides were incubated with rabbit anti-Kdm6a (1:500, Cat #33510S, Cell Signaling Technologies, Danvers, MA), followed by Cy3-labeled goat anti-rabbit (1:1000, Jackson ImmunoResearch) and Hoechst stain to label nuclei. Immunostaining for phosphorylated histone H3 at Ser10 was performed on fresh frozen sections that were fixed in 4% paraformaldehyde and incubated for 20 mins at 98°C in sodium citrate, pH 6.0 prior to cooling for 20 min at room temp and blocking in 0.3% Triton X-100 and 10% NGS in 1X PBS. Slides were incubated with mouse anti-pH3S10 (1:200, Cat #9706S, Cell Signaling) was used at 1:500, followed by Cy2-labeled goat anti-mouse (1:1000, Jackson ImmunoResearch) and Hoechst stain to label nuclei. Z-

stack images were taken at 20X on a Leica SP8 confocal microscope. To quantify EGL length and laminar thickness, we took measurements by tracing the appropriate structures using the Fiji plugin, ObjectJ. Number of pH3S10 positive cells were counted along a segment of cerebellar lobule III. Measurements were performed under blinded conditions, by two independent investigators and analyzed using a one-way ANOVA, followed by a Student's unpaired t-test with correction for multiple comparisons.

Western blotting

The cerebellum was removed and dounced in 1.5X SDS sample buffer at a concentration of 100 mg/mL. Samples were boiled for 15 mins, chilled on ice for 10 mins and insoluble material was spun out at 13,000 RPM for 3 min. 15 µL of total cell lysate/sample was run on 8–12% SDS-PAGE gels and transferred to nitrocellulose. Western blots were blocked in 5% milk, 5% NGS and 1X TBST for 1 hr. The following antibodies were used: rabbit anti-Kdm6a (1:1000, Cat #33510S, Cell Signaling Technologies), rabbit anti-H3K27me3 (1:1250, Cat #07-449, Millipore), mouse anti-Histone H3 (1:1500, Cat #05-499, Millipore) and mouse anti-actin (1:10,000, Cat #MAB1501, Millipore). Secondary antibodies were goat anti-rabbit 770 and goat anti-mouse 680 (1:1000, Biotium). Fluorescent immunoreactivity was imaged on a LI-COR Odyssey and quantified using Fiji. Kdm6a, H3K27me3 and Histone H3 were normalized to actin expression in the same lane. H3K27me3 was then normalized to Histone H3 expression in the same condition.

CGN culture

GNPs were isolated and cultured as previously described (Kokubo et al., 2009). Briefly, cerebella from a litter of P7 mice were chopped into small pieces then digested at 37°C for 30 min in 10 U/ml papain (Worthington), 200 µg/ml l-cysteine (Sigma), and 250 U/ml DNase (Sigma) in PBS. The tissue was mechanically triturated in PBS with trypsin inhibitor and 250 U/ml DNase then centrifuged and resuspended in PBS/BSA. The cell suspension was passed through a cell strainer (Becton Dickinson), layered on a step gradient of 35% and 65% Percoll (Sigma), and centrifuged at 2,500 rpm for 12 min at 25°C. GNPs were harvested from the 35/65% interface, washed in PBS/BSA, resuspended in neurobasal medium with B27 supplements and 2% FBS and plated on poly-d-lysine-coated dishes. In certain cases, as noted in the text, Brain-Derived Neurotrophic Factor (Peprotech) was added at 50 ng/ml on DIV4.

Lentiviral-mediated RNA Interference

For RNA interference, we used two independent shRNAs targeting non-overlapping sequences in mouse *Kdm6b* that were cloned in the lentiviral vector pLKO.1 (Thermo Scientific). *Kdm6b* shRNA1 (TRC#0000095265): CTGTTCTTGAGGGACAACTC. *Kdm6b* shRNA2 (TRC#0000095266): GTTCGTTACAAGTGAGAACTC. Empty pLKO.1 vector was used as the control. Viral shRNAs were packaged as lentivirus in HEK293T cells (ATCC). Neurons in culture were infected with virus at a multiplicity of infection of 1 on DIV1.

Quantitative RT-PCR

RNA was harvested using the Absolutely RNA kit (Agilent). 800 ng of RNA was used for reverse transcription with oligo-dT primers and Superscript II (Invitrogen). Quantitative SYBR green PCR was performed on an ABI 7300 real-time PCR machine (Applied Biosystems) using intron-spanning primers (IDT). Each sample was measured in triplicate, and all data were normalized to the expression of the housekeeping gene *Gapdh* as a control for RNA quantity and sample processing. Primer sequences were as follows: *Dcx*, Fwd-TCGTAGTTTTGATGCGTTGC, Rev- TGCGAATGATGGTCACAAGT; *Gabra1*, Fwd-CCCAATGCACCTGGAAGACT, Rev- AAACGTGACCCATCTTCTGC; *Gabra6*, Fwd-TCTCCCTGGCTCTTCATTA, Rev-AAGTCTGGCGGAAGAAAACA; *Gapdh* Fwd-CATGGCCTTCCGTGTTCT, Rev-TGATGTCATCATACTTGGCAGGTT; *Grin2a* Fwd-TACTCCAGCGCTGAACATTG, Rev-TCAGCTGGACCTGTGTCTTG; *Grin2b* Fwd-GAGCATAATCACCCGCATCT, Rev-AAGGCACCGTGTCCGTATCC; *Grin2c* Fwd-GCAGAACTTCCTGGACTTGC, Rev-CTCTTCACGGGAGCAGTAGG; *Kdm6a* Fwd-AAGGCTGTTCCGTGCTACG, Rev-GGATCGACATAAAGCACCTCC; *Kdm6b* Fwd-ACCACCATCGCTAAATACGC, Rev-CTCCTGTAGCTGTGGCTTCC; *Tiam1* Fwd-CAACACGCTTCCCTGTAGGA, Rev-GAGTTGGTGGCATTGGATCT; *Tubb5* Fwd-ACCGAAGCTGAGAGCAACAT, Rev-CACCATTTACCCCAATGAG.

Neurosphere culture

GNPs were isolated as described above and infected with virus following the protocol of (Zhou et al., 2007) with modifications. Briefly, dissociated CGN progenitors from P4 mice were resuspended in virus-containing medium [DMEM/F12 (1:1), N2 supplement (Invitrogen), 24mM KCl, 6mg/ml glucose, and penicillin/streptomycin (Gibco)] with 0.4 ug/ml polybrene (Sigma) and 25% Shh-N supernatant in an uncoated tissue culture plate. 72 hrs later, aggregate cultures were transferred to media that supports neuronal differentiation (BME (Gibco), 10% Bovine calf serum (Hyclone), N2 supplement (Invitrogen), and penicillin/streptomycin (Gibco)) and kept in this culture for 2 days (DIV5) or 6 days (DIV9) prior to processing for RNA. Shh-N supernatant was generated by transfecting HEK-293T cells with Shh-N-expressing plasmid and harvesting supernatant after 3 days (Fogarty et al., 2007). Thymidine incorporation assay was used to test the efficacy of Shh.

RNA sequencing

CGN cultures infected with pLKO.1 control or Kdm6b shRNA expressing lentiviruses were grown in the presence of 50ng/mL BDNF. RNA was extracted on DIV7, RNA-Seq libraries were generated by the Duke University sequencing core facility and subjected to 50bp single-end Illumina Hi-Seq sequencing. These data were deposited at the Gene Expression Omnibus (GEO datasets accession number pending). Hiseq 4000 reads were trimmed (Trimmomatic), aligned to the mm9 genome (TopHat2), and counted for each gene (htseq-count) (Anders et al., 2015; Bolger et al., 2014; Kim et al., 2013). Read counts were analyzed by edgeR using DESeq2's independent filtering method to determine the cutoff for the minimum read counts per sample (Anders and Huber, 2010; Robinson et al., 2010). Reads counts were also reanalyzed at DIV3-DIV7 timepoints from previous sequence runs (GEO accession GSE60731) using similar methods. For Kdm6b analysis, the model that

edgeR tested included terms for batch effects between the replicates since they were not all generated at the same time. *Kdm6b* genes that were differentially expressed (FDR<0.05) and expressed at biologically relevant levels (absolute logFC>0.5 and RPKM>5) and had a significant DIV3-DIV7 effect were selected for use in the correlation analysis. The R lm() and summary() functions were used to compute the R² and p value for the correlation (Trapnell et al., 2010). Gene sets were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (<http://david.abcc.ncifcrf.gov>) (Huang et al., 2008). We considered as significant those biological pathways and cellular components with Benjamini-corrected p < 0.05.

CGN survival assay

Survival assay was modified from (Bonni et al., 1999). Briefly, GNPs were isolated as described above and resuspended in BME (Gibco) containing calf serum (Hyclone) and membrane-depolarizing concentrations of KCl (25mM) and cultured on German glass coverslips (Bellco). CGNs on glass coverslips were transfected by calcium phosphate (Lyons et al., 2016) after DIV2 with either of the *Kdm6b* shRNAs or the control pLKO lentiviral plasmids together with a plasmid encoding GFP. After DIV5, cells on coverslips were washed and either returned to conditioned medium (complete media), placed in low KCl (5mM) medium deprived of serum (minimal media), or placed in medium without serum but with added 50 ng/mL BDNF for 12, 18, or 24 hrs. Cultures were fixed with paraformaldehyde and subjected to indirect immunofluorescence. Cell survival was assessed in GFP positive neurons based on nuclear morphology visualized using the DNA dye bisbenzimidazole (Hoechst 33258, Sigma). Cell counts were carried out blind to condition and analyzed by Student's unpaired t-test. Approximately 150 cells from 3 coverslips were counted per experiment.

Statistics

Unless otherwise indicated, all data presented are the average of at least three measurements from each of at least two independent experiments. Data were analyzed by ANOVA or Student's unpaired t-test as indicated in the text. Bar graphs show mean values and error bars show S.E.M. In most cases, a p<0.05 was considered significant, however for sequencing data we considered FDR<0.05 significant to correct for multiple comparisons, and for gene pathways we used Benjamini corrected p<0.05 for significance.

Results

Kdm6b expression is induced in newborn CGNs of the postnatal mouse cerebellum

We performed *in situ* hybridization to determine the expression pattern of *Kdm6b* mRNA in the developing mouse cerebellum. At postnatal day 7 (P7), all of the stages of CGN differentiation can be observed within a single parasagittal section (Fig. 1A). Hybridization with an antisense probe against *Kdm6b* at P7 showed that *Kdm6b* mRNA is found in both the EGL and IGL of the developing cerebellum (Fig 1B–C). This *in situ* signal is specific, because it was not observed upon hybridization with the corresponding sense probe (Fig. 1D). Within the EGL, expression of *Kdm6b* is most robust in the inner layer where it does

not colocalize with immunostaining for the dividing cell marker Ki67, which is concentrated in the outer EGL where granule neuron progenitors are found (Fig. 1B–C, E–H).

To determine if there is developmental regulation of expression of the Kdm6 family of histone demethylases over time as CGNs differentiate, we performed *in situ* hybridization for *Kdm6a* and *Kdm6b* in cerebellum across three developmental time points, P0, P7 and P60. We compared *in situ* signal for *Kdm6a* and *Kdm6b* in the outer EGL at P0 and P7 and found no significant difference in signal intensity for either factor (Fig 1I–O; n=3 brains/age, $p>0.1$ *Kdm6a* P0 vs. P7; $p>0.7$ *Kdm6b* P0 vs. P7). Quantitative analysis of *Kdm6b* mRNA expression in the inner EGL compared with the outer EGL at P7 showed that *Kdm6b* expression was induced 1.8 ± 0.029 fold in the inner EGL compared with the outer EGL (Fig. 1M–P). By contrast, *Kdm6a* expression did not change between these two layers (Fig. 1P, ratio *Kdm6a in situ* in inner EGL/outer EGL = 1.03 ± 0.034 ; n=3; $p<0.0001$ fold induction *Kdm6b* across EGL vs. *Kdm6a*). Finally, we compared signal intensity in the P60 IGL to the IGL at P7. Although both *Kdm6a* and *Kdm6b* were detected in the IGL at both ages, the levels of *Kdm6b* were significantly lower in the IGL of the adult brain whereas expression of *Kdm6a* showed no significant change across this time (Fig. 1M–O, Q–T; n=3 brains/age, $p=0.002$ *Kdm6b* P7 vs. P60; $p=0.471$ *Kdm6a* P7 vs. P60). Taken together, these data show that only *Kdm6b*, and not *Kdm6a*, undergoes dynamic and transient upregulation of expression in newly postmitotic CGNs of the developing mouse cerebellum. These data raise the possibility that Kdm6b may have important functions in postmitotic stages of CGN differentiation and maturation.

Neither Kdm6a nor Kdm6b are required for anatomical development of the cerebellum

Previous studies have shown that constitutive knockout of *Kdm6b* disrupts prenatal cerebellar development and results in reduced foliation, which is consistent with impaired GNP proliferation (Shi et al., 2014). However, because Kdm6b is expressed in multiple cell types in the cerebellum including CGNs, Bergmann glia, and Purkinje cells (Mellen et al., 2012), it is not clear whether the defects in cerebellar development detected in the total knockout mice may derive from loss of Kdm6b in CGNs or other cell types. Furthermore, because constitutive *Kdm6b* knockout mice die at birth (Burgold et al., 2012; Satoh et al., 2010), whether Kdm6b plays additional roles in the later stages of CGN differentiation and maturation has remained unknown.

To address these questions, we generated CGN lineage-selective conditional *Kdm6b* knockout mice, by crossing a loxP-flanked *Kdm6b* allele (Shpargel et al., 2014) to mice bearing the *Atoh1*-Cre transgene (Yang et al., 2008). *Atoh1* is an essential fate determining transcription factor for the CGN lineage (Ben-Arie et al., 1997), and Cre recombinase driven by the *Atoh1* transgene is highly expressed in GNPs of the EGL beginning at E14.5 and persisting through the first three week of postnatal life (Yang et al., 2008). In addition to GNPs, the *Atoh1*-Cre transgene is also expressed in a subset of other hindbrain-derived neurons (Wang et al., 2005). However, importantly for this study, it does not drive recombination in other cell types within the cerebellar cortex (Yang et al., 2008). We confirmed that the *Atoh1*-Cre was robustly active in the cerebellar cortex of our mice by visualizing Cre-dependent induction of a *R26-lacZ* reporter (Soriano, 1999) crossed into the

floxed *Kdm6b* strain (Fig. 2A–B). Overall, quantitative RT-PCR from homogenates of total cerebellar cortex harvested at P14 showed a nearly ~80% reduction in total cerebellar *Kdm6b* mRNA expression in *Atoh1*-Cre conditional knockout brains (*Kdm6b* cKO) compared with floxed controls (Ctrl) (Fig. 2C; n=6 control and 5 cKO mice; p=0.001 control vs cKO for *Kdm6b*).

To confirm that *Atoh1*-Cre recombination led to loss of *Kdm6b* in GNP and CGN of the conditional knockout strain, we performed quantitative *in situ* hybridization on Ctrl and *Kdm6b* cKO brains at P7 using a custom RNAscope probe that targets the floxed region (Fig. 2D–K). These data showed a quantitative reduction in *Kdm6b* mRNA signal in both the EGL and IGL consistent with the loss of *Kdm6b* from GNP and CGN (Fig. 2K). ANOVA showed a significant difference of cerebellar cortex layer ($F_{2,17}=24$, p=0.0001) and *Kdm6b* genotype ($F_{1,17}=48$, p<0.0001), but no layer x genotype interaction ($F_{2,17}=6$, p=0.16). As expected, no differences in *Kdm6b* expression were observed comparing expression in the cortex between the floxed control and conditional knockout brains (Fig. 2K; n=3 mice/genotype, p=0.66 Ctrl vs *Kdm6b* cKO in cortex), consistent with the absence of *Atoh1*-Cre expression in this brain region (Fig. 2B). Because *Atoh1*-Cre expression is restricted among cerebellar cell types to GNP and CGN, we assume that the persistent *Kdm6b* signal in cerebellum of the conditional knockouts arises from other cell types, which may include Purkinje cells, astrocytes, and microglia. Indeed, there was extensive overlap of *Kdm6b* mRNA signal in the inner molecular layer of the conditional knockout mice with *in situ* signal for *Pvalb*, a marker of the Purkinje cells, which localize to this layer (Fig. 2I–J). The only cerebellar region where we did detect *Kdm6b* mRNA in the inner EGL of the conditional knockout mice was in cerebellar lobules IX and X of the posterior cerebellar cortex (Fig. 2L–O), which is consistent with the previously reported lack of early postnatal *Atoh1*-Cre activity in these lobules (Pan et al., 2009; Wagner et al., 2017). Taken together, these data validate the loss of *Kdm6b* in the GNP and CGN of the majority of the cerebellar cortex in the conditional knockout mice.

In order to test for potential functional redundancy between *Kdm6b* and its family member *Kdm6a*, we also generated double *Kdm6a* and *Kdm6b* conditional knockouts in GNP by crossing mice bearing both floxed *Kdm6a* and *Kdm6b* alleles to *Atoh1*-Cre (*Kdm6a/b* cKO). Of note, there is no compensatory upregulation of *Kdm6a* expression in single *Kdm6b* cKO mice, and in fact *Kdm6a* levels are also significantly reduced (Fig. 2C, n=6 control and 5 cKO mice, p=0.021 for *Kdm6a* in Ctrl vs cKO). To validate the loss of *Kdm6a* in the double cKO mice, we used an antibody against *Kdm6a* to stain P7 cerebellar sections (Fig. 2P–Q). These data show complete loss of *Kdm6a* from the EGL and partial loss from the IGL, with persistent expression in morphologically identifiable Purkinje cells at the ML/IGL border. Similar to *Kdm6b*, we assume that the expression of *Kdm6a* in the IGL of the cKO mice is found in non-neuronal cells such as astrocytes and microglia. Consistent with these immunostaining data, homogenates of total cerebellar cortex harvested at P7 showed a nearly ~60% reduction in total cerebellar *Kdm6a* protein expression as measured by western blotting (Fig. 2R–S; n=2 Ctrl and 3 *Kdm6a/b* cKO mice; p=0.05 Ctrl vs cKO for actin normalized *Kdm6a*). Notably, we found no difference in global H3K27me3 methylation levels as measured by quantitative western blotting of cerebellar lysates from *Kdm6a/b* cKO mice compared with their littermate controls (Fig. 2T–U, p=0.88), although these data do not

rule out the possibility that *Kdm6a* and *Kdm6b* may locally regulate H3K27me3 levels at specific target genes.

Cells bearing the exons deleted in our conditional *Kdm6b* allele have previously been reported to be null for *Kdm6b* protein (Manna et al., 2015). However, unlike constitutive deletion of *Kdm6b*, which was found to result in defective cerebellar foliation at E18.5 (Shi et al., 2014), we found that *Atoh1*-Cre mediated conditional deletion of *Kdm6b* did not disrupt gross cerebellar morphology as determined by visualizing the foliation pattern of the cerebellum at P7 (Fig. 2B, Fig. 3A–B). Furthermore, double conditional *Kdm6a* and *Kdm6b* knockouts showed normal cerebellar morphology at this age as well (Fig. 3C). To more precisely quantify cerebellar development in each strain, we measured the total distance of the EGL around all of the lobules as an indication of the size of the GNP pool, and we measured the thickness of each of the cerebellar layers (EGL, ML, and IGL) as an indication of the progression of CGN neurogenesis. These data revealed no significant differences between control, *Kdm6b* conditional knockouts, and *Kdm6a/Kdm6b* double conditional knockout mice for either measure (Fig. 3D–E; $n=3$ Ctrl, 2 *Kdm6b* cKO, 3 *Kdm6a/b* cKO mice. For EGL length, one-way ANOVA $F_{2,7}=0.044$, $p=0.96$. For EGL thickness $F_{2,7}=2.96$, $p=0.14$, for ML $F_{2,7}=4.09$, $p=0.09$ and for IGL $F_{2,7}=0.07$, $p=0.93$). To test specifically for the number of proliferating progenitors in the outer EGL at P7, we stained for Ser10 phosphorylated histone H3, which is a marker of mitotic cells. Consistent with our morphological data, we found no significant difference in the number of pH3S10+ nuclei within lobule III of P7 mouse cerebellum comparing control, *Kdm6b* cKO and *Kdm6a/b* cKO mice (Fig. 3F–I, $n=3$ Ctrl, 2 *Kdm6b* cKO, 3 *Kdm6a/b* cKO, $p=0.344$ by one-way ANOVA). Although we cannot rule out that there may be cerebellar defects in these conditional knockout mice at prenatal stages of development, we conclude that *Kdm6a* and *Kdm6b* are not required in GNPs or CGNs for proper anatomical development of the postnatal mouse cerebellum.

Kdm6b regulates a program of mature CGN gene expression

Morphological development of the cerebellum is perturbed by disruption of genes or environmental factors that control GNP division, survival, and differentiation to CGNs (Aruga et al., 2002; Ben-Arie et al., 1997; Pan et al., 2009). By contrast, disruption of genes that contribute to the postmitotic stages of CGN maturation often result in more subtle anatomical changes that in many cases are not immediately apparent (Kadotani et al., 1996; Kokubo et al., 2009). However, these later stages of neuronal maturation are marked by specific gene expression programs, which can easily be followed to monitor the integrity of the CGN maturation process. Previously, we performed RNA-seq at three stages of mouse cerebellar development (P7, P14, and P60) to identify programs of developmentally regulated cerebellar genes (Frank et al., 2015). Cluster analysis revealed four temporally regulated patterns of changing gene expression across this timeframe, each of which was comprised of genes with distinct functional classifications. Because P14 represented a dynamic flexion point for each of the four gene regulatory patterns, to determine whether *Kdm6b* plays a role in the developmental regulation of gene transcription, we sampled a series of developmentally regulated gene products in RNA derived from cerebellar cortex homogenates of control and *Kdm6b* cKO mice at P14.

We first tested a series of gene products that are most highly expressed in immature CGNs and decrease over developmental time: the microtubule binding pro-migratory factor *Dcx*, the immature NMDA receptor subunit *Grin2b*, and the neuron-specific β -tubulin subunit *Tubb5*. We found that the mRNA expression levels of these genes were not significantly different between control and *Kdm6b* cKO cerebellum (Fig. 4A, n=6 control and 5 cKO mice, p=0.69 for *Dcx*, p=0.22 for *Grin2b*, p=0.48 for *Tubb5*, all for Ctrl vs cKO). We then quantified expression of a set of genes that show increased expression as CGNs mature: the mature GABA_A receptor subunits *Gabra1* and *Gabra6*, the mature NMDA receptor subunit *Grin2c* and the Rac GEF *Tiam1*. All of these genes showed reduced mRNA expression in the *Kdm6b* cKO cerebella relative to control, with all but *Gabra1* showing statistically significant reductions (Fig. 4B, n=6 control and 5 cKO mice, p=0.16 for *Gabra1*, p=0.048 for *Gabra6*, p=0.007 for *Grin2c* and p=0.008 for *Tiam1*, all for Ctrl vs cKO). Thus, these data suggest that *Kdm6b* is required for the induction of at least a subset of genes that define the mature CGN phenotype.

To confirm that these *Kdm6b*-dependent changes in gene expression were occurring in CGNs and to more precisely determine the timing of *Kdm6b* action, we turned to an *ex vivo* system in which we could control the temporal maturation of CGNs. Specifically, we employed an established protocol (Zhou et al., 2007) in which we isolated GNPs from the developing P4 cerebellum, held them in the cell cycle by culturing them in the presence of sonic hedgehog (Shh), used lentiviral infection of shRNAs on the first day *in vitro* (DIV1) to knockdown *Kdm6b* expression (KD), and then on DIV4, switched the cells to a differentiation medium at which time they undergo concerted differentiation (Fig. 4C). Compared with cells infected with a control lentiviral plasmid, we saw robust knockdown of *Kdm6b* expression by DIV5 with either of two independent shRNAs targeting *Kdm6b* (Fig. 4D, n=4 Ctrl, 4 shRNA1, 2 shRNA2 at each DIV from 2 independent experiments, p=0.03 shRNA DIV5, p=0.001 shRNA2 DIV5, p=0.007 shRNA1 DIV9, p=0.04 shRNA2 DIV9, all vs Ctrl on the same DIV). When we compared the expression of genes from the early and late gene expression programs from Fig 4A–B between DIV5 and DIV9, we found that whereas the early genes drop their expression only slightly over this time frame, the late genes were significantly induced (Fig. 4E, n=4 at each age, p=0.11 for *Dcx*, p=0.65 for *Grin2b*, p=0.19 for *Tubb5*, p=0.0005 for *Gabra1*, p=0.0001 for *Gabra6*, p=0.0001 for *Grin2c*, p=0.006 for *Tiam1*, all DIV9 vs DIV5). Thus, this culture system provides a useful model for studying the requirement for *Kdm6b* in developmental upregulation of this mature CGN gene program.

Similar to our observations in P14 *Kdm6b* cKO cerebellum, we found overall that induction of the late gene expression program was reduced in neurons lacking *Kdm6b* relative to control, although some of the effects showed only trends whereas others reached significance (Fig. 4F, n=4 Ctrl, 4 shRNA1 and 2 shRNA2, p=0.0032 for shRNA1 and 0.042 for shRNA2 vs Ctrl for *Gabra1*, p=0.17 for shRNA1 and 0.11 for shRNA2 vs Ctrl for *Gabra6*, p=0.0001 for shRNA1 and 0.0056 for shRNA2 vs Ctrl for *Grin2c*, and p=0.02 for shRNA1 and 0.26 for shRNA2 vs Ctrl for *Tiam1*). The failure to fully induce the late gene expression program was not due to a selective loss of neurons in the culture over time because expression of the neuronal marker *Tubb5* was not reduced upon knockdown of *Kdm6b* at either the early or late time point (Fig. 4G).

To determine whether dysregulation of these specific genes reflects a broader role for Kdm6b in the regulation of the gene expression program induced during CGN maturation, we cultured control and *Kdm6b* KD CGNs and used RNA harvested from three independent replicate pairs at DIV7 for RNA sequencing (**Table S1**). Analysis of the gene expression profiles from control compared with knockdown revealed 1556 genes with significantly differential expression at $FDR < 0.05$ that were expressed at biologically relevant levels (absolute $\log FC > 0.5$ and $RPKM > 5$; **Table S2**). To determine how this set of Kdm6b-regulated genes relates to the changes in gene expression that occur during CGN maturation, we assessed the correlation between these Kdm6b-dependent genes and the set of 353 genes that show significant changes in expression from DIV3 to DIV7 in culture, as identified in RNAseq data we generated in a previous study (Frank et al, 2015) (**Table S2**). As shown in Fig. 5, we found that genes showing reduced expression upon *Kdm6b* knockdown are highly likely to be those that increase expression from DIV3 to DIV7 as CGNs mature, whereas genes that show increased expression in Kdm6b KD neurons are likely to be reduced in expression over this period of maturation. The correlations in the expression of these genes revealed an R^2 of 0.463 ($p < 2.2e^{-16}$), which indicates that 46% of the variation with Kdm6b knockdown can be explained by DIV3-DIV7 gene expression.

Gene ontology (GO) analysis of the 246 developmentally upregulated genes that are reduced upon Kdm6b KD (**Table S3**, lower right quadrant of Fig. 5) showed significant enrichment for the three biological process categories of cell adhesion (GO:00007155), extracellular matrix organization (GO:0030198), and negative regulation of cell proliferation (GO:0008285). Multiple cellular component categories were significantly enriched in this set, including extracellular matrix and cell surface consistent with the biological process categories. The synapse category was also significantly enriched (Benjamini corrected $p = 5.8e^{-4}$). The 19 Kdm6b-sensitive and developmentally upregulated genes in the synapse category (Table 1) include neurotransmitter receptors as well as both pre- and post-synaptic gene products. Taken together, these data suggest that Kdm6b is selectively required for developmental induction of a gene expression program that turns on relatively late during the process of CGN differentiation and contributes to aspects of functional synaptic maturation.

Kdm6b is required for BDNF-dependent induction of CGN gene expression

Brain-Derived Neurotrophic Factor (BDNF) is an important regulator of cerebellar development, playing key roles in promoting CGN survival (Bonni et al., 1999), migration (Borghesani et al., 2002), and synapse development (Carter et al., 2002). BDNF effects changes in neurons by binding to the receptor tyrosine kinase TrkB, which induces the activation of intracellular calcium and MAP kinase signaling pathways (Deinhardt and Chao, 2014). The targets of these signaling cascades include both cytoplasmic and nuclear proteins that can induce a broad range of changes in neuronal function. Some of the biological effects of BDNF on neuronal function are transcription-dependent (Bonni et al., 1999; Kokubo et al., 2009), and indeed it has been previously reported that addition of BDNF to CGNs in culture promotes induction of a program of gene expression that includes *Tiam1* and *Grin2c* (Sato et al., 2006; Suzuki et al., 2005), two of the genes that we found were sensitive to loss of *Kdm6b*.

We first confirmed that expression of *Grin2c* and *Tiam1* was regulated by BDNF in CGNs in our culture system. Indeed, addition of 50 ng/mL of BDNF to the culture medium we used to differentiate GNPs on DIV4 promoted a small induction of both mRNAs by DIV5 and a significant increase in their expression by DIV9 (Fig. 6A). Knockdown of *Kdm6b* with either shRNA had little effect on expression of *Grin2c* or *Tiam1* at DIV5, but it significantly impaired the ability of BDNF to promote expression of these mRNAs as measured on DIV9 (Fig. 6B, *Grin2c* on DIV9 $p=0.013$ shRNA1 vs. Ctrl, $p=0.015$ shRNA2 vs. Ctrl; *Tiam1* on DIV9 $p=0.03$ shRNA1 vs. Ctrl, $p=0.043$ shRNA vs Ctrl). The failure of BDNF to induce *Tiam1* and *Grin2c* gene expression in *Kdm6b* knockdown neurons was not due to a complete impairment in BDNF/TrkB signaling, because addition of BDNF was still sufficient to promote neuronal survival in both control and *Kdm6b* shRNA transfected CGNs in a serum withdrawal paradigm that has been used extensively to study the pro-survival mechanisms of BDNF signaling (Bonni et al., 1999) (Fig. 6C–G). Specifically, although knockdown of *Kdm6b* leads to a significant increase in death when survival medium is withdrawn (Fig. 6F, $n=8$ independent samples/virus and treatment, $p=0.0059$ Ctrl vs *Kdm6b* shRNA in minimal media), which is consistent with the pro-survival function of *Kdm6b* we identified in hippocampal neurons previously (Wijayatunge et al., 2014), addition of BDNF to the minimal medium promotes survival to a similar degree in both control and *Kdm6b* knockdown neurons (Fig. 6F, $n=8$ /virus and treatment, $p=0.0065$ for Ctrl and $p=0.0087$ for *Kdm6b* shRNA1 in minimal medium vs. BDNF; Fig. 6G, $p=0.81$ Ctrl vs. *Kdm6b* shRNA1). Although we cannot rule out the possibility that loss of *Kdm6b* selectively impairs aspects of the BDNF/TrkB signaling cascade that affect transcription but not survival, our data are still consistent with a model in which *Kdm6b* is required to make *Tiam1* and *Grin2c* transcription permissive for regulation by BDNF.

Discussion

In this study, we report the characterization of CGN differentiation in the cerebellum of *Kdm6b* conditional knockout mice and following *Kdm6b* knockdown in GNPs in culture. Our data show that the expression of *Kdm6b* is not required in GNPs *in vivo* for the generation of CGNs, however *Kdm6b* does contribute to the induction of a late program of gene expression, which includes gene products that regulate synaptic functions in the mature CGN state. These data implicate *Kdm6b* in the late postmitotic stages of neurogenesis, complementing its previously identified functions in earlier steps of neuronal commitment and differentiation.

Our data provide further support for the model that *Kdm6b* promotes differentiation of multiple different cell types (Agger et al., 2007; Kartikasari et al., 2013; Li et al., 2014; Ohtani et al., 2013; Sen et al., 2008; Yang et al., 2013; Ye et al., 2012). Many past studies in the neuronal lineage have focused on relatively early stages of cell fate determination. For example, knocking down *Kdm6b* in embryonic stem cells impairs the developmental induction of neural lineage genes, and in neural stem cells, retinoic acid-induced expression of *Kdm6b* is sufficient to drive the expression of several neuronal genes (Burgold et al., 2008; Jepsen et al., 2007). Endogenous *Kdm6b* is required for the neurogenesis of dorsal interneurons in the embryonic spinal cord and for the formation of new olfactory bulb neurons in the adult CNS (Akizu et al., 2010; Estaras et al., 2012). However, *Kdm6b* has

also been implicated in postmitotic stages of cellular maturation in other cell types. For example, Kdm6b is required for terminal T-cell differentiation (Manna et al., 2015), and Kdm6b promotes macrophage transdifferentiation induced by LPS stimulation (De Santa et al., 2007). In the nervous system, the analysis of respiratory circuits in constitutive Kdm6b knockout mice has suggested that Kdm6b could contribute to the functional maturation of neurons. Kdm6b knockout mice die perinatally due to respiratory failure that is associated with impaired phrenic nerve activity (Burgold et al., 2012). Immunohistochemical analysis of the pre-Bötzinger complex, which controls respiration, revealed an impairment in the knockouts at E18.5 of neurokinin1 receptor/somatostatin positive cells, which are key to the functional output of this nucleus. Interestingly, younger embryos (E14.5–15.5) were capable of generating respiratory movements, thus the authors proposed that the respiratory failure phenotype at E18.5 might arise from failure to mature or maintain neurons in the respiratory circuits of the Kdm6b knockout mice rather than a failure to form these neurons altogether.

Our use of CGNs from the developing postnatal mouse cerebellum in this study now allowed us to assess the functional requirements for Kdm6b at multiple stages of neurogenesis including postmitotic stages of neuronal differentiation. The developmental induction of *Kdm6b* expression in newborn CGNs first suggested to us that Kdm6b might play a role in maturation of these neurons. Indeed, we found reduced expression in the cerebellum of our *Kdm6b* cKO mice of several gene products that are not turned on in CGNs until they reach the IGL and begin to mature (Abe et al., 2011; Ding et al., 2013; Frank et al., 2015). We focused in particular on a set of potential Kdm6b target genes (*Gabra1*, *Gabra6* and *Grin2c*) that encode neurotransmitter receptor subunits. The coordinated regulation of these receptor proteins is essential for functional synaptic transmission and synapse maturation in cerebellar circuits. Genetic disruption of either *Gabra6* or *Grin2c* does not impair morphological aspects of cerebellar development and the loss of these gene products is not associated with generally impaired performance in neurological screens, even though the absence of these ion channel subunits significantly alters inhibitory or excitatory transmission at CGN synapses (Kadotani et al., 1996; Korpi et al., 1999). Given that we find that the loss of *Kdm6b* in CGNs is associated with altered expression of multiple genes whose products are important for the formation and function of CGN synapses, it will be interesting in the future to conduct morphological and electrophysiological analyses of CGN synaptic connectivity in the *Kdm6b* cKO mice and their control littermates to determine the functional impact of these gene expression changes on cerebellar circuit development and function.

Like other chromatin regulatory factors that do not bind directly to DNA, Kdm6b is able to mediate distinct functions in different types of cells by virtue of being recruited to its target genes through interactions with transcription factors. In some cases, these transcription factors are known, for example in Th1 cells Kdm6b binds T-bet to induce *Ifng* expression (Miller et al., 2010) and in the embryonic dorsal nerve cord, Kdm6b interacts with Smad proteins to mediate gene expression induced by BMP signaling (Estarras et al., 2012). However, the transcription factors that interact with Kdm6b in CGNs are not known. One possibility is that the genes we have identified here are direct targets of regulation by Kdm6b. We previously revealed a function for the Zic family of transcription factors in induction of the mature CGN expression program (Frank et al., 2015), and the Zics also

regulate Shh target genes in GNPs (Blank et al., 2011) so this family represents a promising candidate. However, an alternative possibility is that Kdm6b acts indirectly to promote the expression of the CGN maturation program. ChIP-seq to map the binding pattern of Kdm6b across the CGN genome, as has been done for other types of neurons (Estarras et al., 2012), will help to resolve this issue.

Another unanswered question from our study regards the mechanism by which loss of Kdm6b impairs BDNF-dependent induction of gene expression in CGNs. We have shown that BDNF signaling is intact in the presence of Kdm6b, placing the requirement for Kdm6b downstream of BDNF. In several contexts, the link between Kdm6b and stimulus-induced gene expression, such as that promoted by LPS in macrophages or retinoic acid in neural progenitors, has been linked to the ability of these stimuli to induce the expression of *Kdm6b* (De Santa et al., 2007; Jepsen et al., 2007). However BDNF did not induce expression of *Kdm6b* (data not shown) under the culture conditions we used to show the requirements for Kdm6b in the BDNF-dependent induction of *Grin2c* and *Tiam1* in Fig. 6B. In the case of *Grin2c*, BDNF is known to promote transcription via phosphorylation of the transcription factor Etv1, which binds to the *Grin2c* promoter region (Abe et al., 2012). Notably, Etv1 expression is induced as CGNs mature and it is downregulated in Kdm6b KD CGNs (**Table S3**). Examining Etv1 phosphorylation, binding, and function at *Grin2c* in Kdm6b knockout/knockdown CGNs may thus offer mechanistic insights into the convergence of these transcriptional regulatory processes.

One surprising finding of our study is that we did not observe a disruption of cerebellar morphology at P7 in the *Kdm6b* cKO mice, which might have been expected from prior studies that have implicated Kdm6b as a positive regulator of neural stem cell differentiation in general (Jepsen et al., 2007; Park et al., 2014) and GNP proliferation in particular (Shi et al., 2014). Notably, mice bearing a constitutive germline deletion of *Kdm6b* have a small cerebellum with reduced foliation compared with their control littermates at E18.5, which suggests that these mice suffer impaired GNP proliferation (Shi et al., 2014). Sonic hedgehog (Shh) signaling is an important regulator of GNP proliferation in the cerebellum (Leto et al., 2016), and Kdm6b-lacking GNPs cultured in presence of Shh display reduced proliferative rates and impaired Shh-dependent gene expression, indicating that Kdm6b contributes to Shh-dependent GNP proliferation under these conditions (Shi et al., 2014). However, there are additional factors that regulate GNP proliferation *in vivo*, such as Notch2 (Leto et al., 2016), and the fact that we did not find disruption of postnatal cerebellar morphology or the number of mitotic GNPs at P7 in the *Kdm6b* cKO mice suggests that the contribution of Kdm6b to GNP proliferation driven by Shh in isolation is not essential in the complex growth factor milieu that comprises the postnatal developing cerebellum. Additionally, it is known that *Kdm6b* is expressed in multiple cell types in the cerebellum, thus an alternative explanation for the distinct cerebellar phenotypes observed in the constitutive and *Atoh1*-conditional *Kdm6b* knockout strains may arise from functions of Kdm6b in non-CGN lineage cells that subsequently impact GNP proliferation via non-cell autonomous mechanisms. For example, *Kdm6b* mRNA is expressed in Purkinje neurons (Mellen et al., 2012) and it is these neurons that are the source of the endogenous Shh that promotes GNP proliferation (Leto et al., 2016). If Shh production from Purkinje neurons

were to be impaired in the absence of Kdm6b in the constitutive knockouts, this could amplify the consequences of impaired Shh-dependent signaling intrinsic to the GNPs. The fact that we find extracellular matrix as a significant GO category among the genes whose expression is impaired by Kdm6b knockdown (**Table S2–S3**) points toward a potentially important role for Kdm6b in cell-cell communication in the cerebellum. Using the conditional knockout strain to eliminate *Kdm6b* from additional cell types of the cerebellum will be a useful way to comprehensively understand the role of this regulatory factor in brain developmental processes that involve communication between multiple cell types.

Kdm6b was first identified on the basis of its JmjC domain as a histone demethylase and shown to be selective for the removal of di- and tri-methylation on histone H3K27 (Agger et al., 2007; Black et al., 2012; Hong et al., 2007; Lan et al., 2007). Because polycomb complex regulation of H3K27 trimethylation is known to be important for cell development (Margueron and Reinberg, 2011) and because a number of cell-type specific genes induced during differentiation show bivalent promoters marked by both H3K4me3 and H3K27me3 prior to their induction (Bernstein et al., 2006; Mikkelsen et al., 2007; Mohn et al., 2008), it seems plausible that at least some of the capacity for Kdm6b to promote cellular differentiation may be mediated by its ability to remove repressive H3K27me3 from bivalent genes. However, much of the evidence in support of this relationship between Kdm6b and histone demethylation in development is correlational. For example, although the knockdown or knockout of *Kdm6b* is associated with the failure to lose H3K27me3 at a set of putative target genes in differentiating ES cells and neural stem cells (Burgold et al., 2008; Park et al., 2014), it has not been established whether a histone demethylase dead version of *Kdm6b* would be able to rescue these neuronal differentiation phenotypes. We saw no global change in H3K27me3 in cerebellar lysates from *Kdm6a/b* cKO mice (Fig. 2T–U), though this does not rule out the possibility that Kdm6a and Kdm6b may regulate H3K27me3 locally at specific target genes.

Supporting the functional requirement for the histone demethylase action of Kdm6b in CNS development, crossing a histone demethylase dead Kdm6b expressed from a BAC transgene onto the *Kdm6b* knockout background failed to rescue perinatal lethality (Burgold et al., 2012), though this study did not directly assess the impact of transgene expression on neuronal development. However, some of the biological functions that have been ascribed to Kdm6b are distinctly not dependent on or mediated by its histone demethylation domain (Miller et al., 2010). For example, although both Kdm6a and Kdm6b are required for embryonic development in the mouse, they are not required for the concerted loss of H3K27me3 that occurs across the *Hox* gene locus during embryonic development, which is required for organismal patterning (Shpargel et al., 2014). In addition to removing repressive histone marks, Kdm6b has been shown to promote transcription by localizing Set1/MLL components and elongation factors to gene promoters (Chen et al., 2012; Miller et al., 2010; Shi et al., 2014) and it is possible that these functions of Kdm6b outweigh its actions as a histone demethylase in some developmental processes. For the CGN lineage, with respect to the transcriptional effects of Shh on GNPs, which is associated with decreased H3K27me3 at Shh-induced genes, knockdown/replacement studies showed that wildtype but not histone demethylase dead Kdm6b could rescue Shh-induced gene expression, implicating the

demethylase function of Kdm6b in this aspect of GNP biology (Shi et al., 2014). Whether H3K27me3 is downregulated during postmitotic CGN maturation at the genes we identified as Kdm6b-dependent in this study, and whether the histone demethylase function of Kdm6b is required for the induction of these genes in maturing CGNs will be important questions to answer in the future.

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Highlights

- Characterizes developmental expression of the H3K27 demethylases Kdm6a and Kdm6b across multiple stages of cerebellar granule neuron differentiation
- Uses a conditional cerebellar granule neuron selective knockout strategy to demonstrate a requirement for Kdm6b in the maturation but not the formation of cerebellar granule neurons in vivo.
- Identifies a requirement for Kdm6b in BDNF-dependent induction of Grin2c and Tiam1, which play important roles in development and function of synapses.

Figure 1. *Kdm6b* mRNA expression is induced in newborn CGNs of the postnatal mouse cerebellum

A) Layered organization of mouse cerebellum at postnatal day 7. The spatial distribution of the three stages of CGN differentiation (GNPs in the outer EGL, newborn CGNs in the inner EGL, and maturing CGNs in the IGL) as well as the localization of migrating CGNs, Bergmann glia, and Purkinje cells in the ML is displayed. **B)** Antisense *Kdm6b* in situ on a parasagittal section of cerebellum at P7. **C)** Enlargement of the boxed region shown in (B) reveals maximum signal in the inner EGL and IGL. **D)** Sense *Kdm6b* in situ control. **E–H)** TSA-amplified fluorescent in situ hybridization (F, red) for *Kdm6b* combined with immunostaining for the dividing GNP marker Ki67 (G, green). Nuclei are labeled with Hoechst in blue (E) to show cerebellar layers in the merge (H). Dotted white lines show the boundaries between outer EGL, inner EGL, ML, and IGL. Quantitative double fluorescent *in situ* for *Kdm6a* (I, green) and *Kdm6b* (J, red) in lobule III of the cerebellum at P0 (I–K), P7 (M–O) and P60 (Q–S). *In situ* signal intensity within the white boxes was divided by Hoechst signal intensity (K, blue) to control for cell density. Quantified data for the relative *in situ* signal in the EGL at P0 and the outer EGL at P7 are shown in (L), $n=3$ brains, $p>0.1$ *Kdm6a* P0 vs. P7; $p>0.7$ *Kdm6b* P0 vs. P7. Quantified data for relative *in situ* signal of *Kdm6a* and *Kdm6b* in the outer EGL compared with inner EGL at P7 are shown in (P), $n=3$

brains, $*p < 0.05$ *Kdm6b* vs *Kdm6a*. Quantified data comparing IGL signal at P60 with IGL signal at P7 are shown in (T), $n=3$ brains/age, $*p < 0.05$ P60 vs P7 for *Kdm6b*. Scale bars are 250 μm in B, 50 μm in D–K, M–O, Q–S.

Figure 2. Conditional knockout of *Kdm6b* and *Kdm6a* in the CGN lineage with *Atoh1-Cre*
A–B) Whole mount images of *Kdm6b* floxed (A) and *Kdm6b* floxed plus *Atoh1-Cre* (B) brains both with the *Rosa lacZ* transgene at P7 reacted for β -gal activity. C) Quantification of *Kdm6a* and *Kdm6b* mRNA expression by quantitative RT-PCR in cerebellar cortex from P14 control and cKO mice. $n=6$ control and 5 cKO mice. D–K) Quantitative fluorescent *in situ* validation of *Kdm6b* expression in lobule III of control and *Atoh1*-conditional knockout cerebellum (cKO) at P7. *Pvalb* is shown as a marker of Purkinje neurons (J). *Kdm6b* *in situ* signal (E and H), quantification normalized for DAPI intensity to control for cell density is shown in K. * $p<0.05$ *Kdm6b* cKO vs. Ctrl in cerebellar cortical layers. L–O) Fluorescent *in situ* detection of *Kdm6b* expression in lobule IX of *Atoh1*-conditional KO cerebellum (cKO) at P7. P–Q) Immunostaining for Kdm6a (red) in lobule III of the cerebellum at P7 in a control (P) or *Kdm6a* and *Kdm6b* double cKO (Q) brain. Nuclei are labeled with Hoechst in blue. R–S) Western blot quantification of Kdm6a in cerebellum of control and double cKO mice at P7, $n=3$ control and 3 double cKO brains, * $p<0.05$ by Student's unpaired t-test. T–U) Western blot quantification of H3K27me3 levels in cerebellum of control and *Kdm6a/b* cKO mice at P7, $n=2$ Ctrl, 3 *Kdm6a/b* cKO, $p=0.88$ by one-way ANOVA. Scale bars are 2mm in A and B, 50 μ m in D–J, L–O, 100 μ m in P–Q.

Figure 3. Neither *Kdm6a* nor *Kdm6b* is required in CGNs for normal cerebellar morphology at P7

A–C) Representative Hoechst -stained images of double floxed control (A), *Kdm6b* cKO (B), and *Kdm6a/Kdm6b* double cKO (C) cerebellum at P7. D) Quantification of total EGL length (in μm) at P7 from parasagittal sections as in A–C. E) EGL, ML, and IGL thickness in lobule III as measured in the region shown in the white boxes in A–C, n=3 floxed control, 2 *Kdm6b* cKO, and 3 double cKO brains. F) Parasagittal sections of double floxed control (F), *Kdm6b* cKO (G), and *Kdm6a/Kdm6b* double cKO (H) cerebellum at P7, stained with phospho-Histone H3, a marker for mitotic cells (green) and Hoechst to label nuclei (blue). H) Quantification of the number of proliferating cells along a segment of lobule III (white boxes) between double floxed control, *Kdm6b* cKO and *Kdm6a/Kdm6b* double cKO. Scale bar in A, 250 μm and in F, 100 μm.

Figure 4. Loss of *Kdm6b* in GNPs disrupts a program of gene expression associated with CGN maturation

A–B) Quantitative RT-PCR measurement from cerebellar cortex from P14 control and cKO mice of a set of genes that are predominantly expressed in early (A) or late (B) CGN differentiation as reported in Frank et al., 2015. Data are graphed as normalized relative to levels in control mice. n=6 control and 5 cKO. *p<0.05 cKO vs. control mice. C) Diagram of the *ex vivo* experimental paradigm. D) Quantitative RT-PCR validation of *Kdm6b* knockdown with two independent shRNAs, n=4 Ctrl, 4 shRNA1, 2 shRNA2 from two independent experiments, *p<0.05 *Kdm6b* KD compared with pLKO control virus. E) qRT-PCR for the early and late expressed genes from A–B over the time course shown in C in control-infected neurons, orange bars represent DIV5, green bars represent DIV9, n=4 at each age, *p<0.05 DIV9 vs. DIV5. Data are graphed relative to levels at DIV5. F) qRT-PCR on DIV9 for the late expressed genes from B in neurons infected with control lentivirus (Ctrl) or shRNAs targeting *Kdm6b*. Data are graphed relative to Ctrl infected cells on DIV5 (from E), n=4 Ctrl, 4 shRNA1, 2 shRNA2, *p<0.05 shRNA vs. Ctrl. G) qRT-PCR for the neuronal marker *Tubb5* in lentiviral infected neurons on DIV5 and DIV9, n=4 Ctrl, 4 shRNA1, 2 shRNA2, legend as in F.

Figure 5. RNA-seq identification of the Kdm6b-sensitive program of CGN maturation genes
 Relationship between gene expression during maturation of cultured CGNs and expression effects of *Kdm6b* shRNA knockdown for genes marked significant in *Kdm6b* knockdown RNA-seq experiments (FDR < 0.05, n=3 independent cultures). X-axis shows log_2 fold change in gene expression between +3 DIV and +7 DIV in cultured CGNs (Frank et al 2015). Y-axis shows log_2 fold change in gene expression between control infected and *Kdm6b* knockdown. The relationship is fit by a negative Pearson correlation coefficient (r).

Figure 6. Knockdown of *Kdm6b* impairs the BDNF-dependent induction of *Grin2c* and *Tiam1* expression in CGNs

A) qRT-PCR for *Grin2c* and *Tiam1* in CGNs grown as in Figure 4C using either CGN medium (black) or medium supplemented with 50 ng/mL BDNF (green) starting at DIV4. n=2/condition, *p<0.05. B) qRT-PCR of neurons treated with 50 ng/mL BDNF starting on DIV4 and harvested on DIV5 and DIV9. Neurons were infected with control lentivirus (Ctrl) or shRNAs targeting *Kdm6b*. Data are graphed relative to Ctrl infected cells on DIV5, n=2 Ctrl, 2 shRNA1, 2 shRNA2, *p<0.05 shRNA vs. Ctrl. C) Images of CGNs in culture with pyknotic nuclei (red arrows) and healthy nuclei (white arrow) identified by Hoechst staining. D) GFP indicates cells transfected with control or Kdm6b shRNA plasmids (green arrows). Scale bar in C, 25 μ m. E) Quantification of cell death over time in complete media (black), following total growth factor withdrawal (minimal media, gray), or upon transfer to minimal media with 50 ng/ml BDNF added (green). n=3 independent samples/time point and condition, *p<0.05 BDNF treatment compared with minimal medium over the time course. F) Quantification of cell death in control and Kdm6b shRNA transfected CGNs at 24 hrs after change to the indicated medium, n=8 independent experiments/virus infection and condition, *p<0.05 for Kdm6b shRNA vs Ctrl in minimal medium, #p<0.05 for Ctrl or

Kdm6b shRNA1 in BDNF vs minimal medium. G) Quantification of the percent cell survival induced by BDNF addition to minimal media from the data shown in (F).

Table 1

Genes in the “synapse” cellular compartment category that are significantly downregulated by Kdm6b knockdown and upregulated over CGN maturation in culture.

Gene	Common Name	Synaptic Function
<i>Atp2b2</i>	Ca ⁺⁺ transporting plasma membrane ATPase	Intracellular calcium signaling
<i>Atp1a2</i>	Na ⁺ /K ⁺ , ATPase alpha 2 polypeptide	Membrane potential
<i>Tiam1</i>	T cell lymphoma invasion and metastasis 1	Rac GEF, dendrite and spine development
<i>Dlg1</i>	Discs Large homolog 1	PDZ domain synaptic scaffold protein
<i>Dact1</i>	Dishevelled-binding antagonist of beta-catenin 1	Regulator of Wnt/beta-catenin signaling
<i>Doc2b</i>	Double C2 beta	Calcium sensor for neurotransmitter release
<i>Frrs1l</i>	Ferric-chelate reductase 1 like	AMPA receptor outer core associated protein
<i>Gabrd</i>	GABA-A receptor subunit delta	GABA receptor
<i>Gad1</i>	Glutamate decarboxylase 1	GABA synthetase
<i>Gria1</i>	Ionotropic glutamate receptor subunit GluA1	AMPA-type glutamate receptor
<i>Grin2c</i>	Ionotropic glutamate receptor subunit GluN2C	NMDA-type glutamate receptor
<i>Olfm3</i>	Olfactomedin 3	AMPA receptor clustering
<i>Rims3</i>	Regulating synaptic membrane exocytosis 3	Synaptic vesicle exocytosis
<i>Sspn</i>	Sarcospan	Dystrophin-glycoprotein adhesion complex
<i>Slc17a7</i>	Solute carrier family 17 a7 (VGlut1)	Vesicular glutamate transporter
<i>Sv2c</i>	Synaptic vesicle glycoprotein 2c	Synaptic vesicle protein
<i>Synpo</i>	Synaptopodin	Actin-regulating dendritic spine protein
<i>Sdc2</i>	Syndecan 2	Heparin sulfate proteoglycan, adhesion
<i>Tnfrsf1a</i>	Tumor necrosis factor alpha receptor 1	TNFA signaling at synapses