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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.

1. 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) 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 is 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

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

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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 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 linages 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.

2. Methods and materials

2.1. 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.

2.2. 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 (Fisher). 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′- GTCGACCAT CGGGCAGTGACCCTC- 3′; *Kdm6b* antisense probe 5′- GGATCCGACCT TGGCTCTGTGCTGAC- 3′. Hybridized riboprobes were visualized by immunological detection with alkaline phosphatase (AP)-conjugated anti-DIG antibodies (Roche) and developed using 5-bromo-4-chloroindolylphosphate/nitroblue tetrazolium (BCIP/NBT; Roche). Images were captured on a Leica DMI4000 inverted fluorescence microscope at 10 × 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; *Pv*alb (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 DAPI (Cat #320858) to label nuclei of all cells for identification of anatomical landmarks, and Z-stack images were taken at 40 × on a Leica SP8 confocal microscope and analyzed in Fiji.

2.3. 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 10 × 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 min prior to blocking in 0.3% Triton X-100 and 10% NGS in 1 × 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 min 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 1 × 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 20 × on a Leica SP8 confocal microscope. To quantify EGL length and laminar thickness, we