

Published in final edited form as:

Cell Rep. 2014 September 11; 8(5): 1290–1299. doi:10.1016/j.celrep.2014.07.060.

Epigenetic Activation of Neuronal Gene Expression by JMJD3 is Required for Postnatal and Adult Brain Neurogenesis

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SUMMARY

The epigenetic mechanisms that enable lifelong neurogenesis from neural stem cells (NSCs) in the adult mammalian brain are poorly understood. Here we show that JMJD3, a histone H3-lysine 27 (H3K27) demethylase, acts as a critical activator of neurogenesis from adult subventricular zone (SVZ) NSCs. JMJD3 is upregulated in neuroblasts, and *Jmjd3*-deletion targeted to SVZ NSCs in

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AUTHOR CONTRIBUTIONS

D.H.P. and D.A.L. conceived of the project, designed experiments, analyzed data, and wrote the manuscript. D.H.P., S.J.H., R.D.S., S.J.L., and S.W.S. performed experiments and analyzed data. J.S. and G.T. performed preliminary ChIP experiments and contributed JMJD3 antibodies. M.M.M. and N.I. contributed transgenic mice. All authors helped write and edit the manuscript.

both developing and adult mice impairs neuronal differentiation. JMJD3 regulates neurogenic gene expression via interaction at not only promoter regions, but also neurogenic enhancer elements. JMJD3 localizes at neural enhancers genome-wide in embryonic brain, and in SVZ NSCs, JMJD3 regulates the I12b enhancer of *Dlx2*. In *Jmjd3*-deleted SVZ cells, I12b remains enriched with H3K27me3, and *Dlx2*-dependent neurogenesis fails. These findings support a model in which JMJD3 and the poised state of key transcriptional regulatory elements comprise an epigenetic mechanism that enables the activation of neurogenic gene expression in adult NSCs throughout life.

INTRODUCTION

Epigenetic regulation via histone methylation is critical for the establishment and maintenance of lineage-specific gene expression. The trimethylation of histone 3 at lysine 27 (H3K27me3) by Polycomb Repressive Complex 2 (PRC2) correlates with transcriptional repression (Margueron and Reinberg, 2011), and such gene repression is critical for the proper function of stem cells in both the embryonic and adult brain (Hirabayashi and Gotoh, 2010). While previous studies have highlighted the importance of H3K27me3 placement and gene repression in neural development (Hirabayashi et al., 2009; Hwang et al., 2014; Pereira et al., 2010), how H3K27me3 is removed for the activation of neurogenic gene expression is not well understood.

JMJD3 (KDM6B) is an H3K27me3-specific demethylase that belongs to the family of JmjC domain-containing proteins (Agger et al., 2007; De Santa et al., 2007). *Jmjd3*-null mice die at birth due to respiratory failure (Burgold et al., 2012; Satoh et al., 2010). In the embryonic mouse forebrain, *Jmjd3* expression is regulated (Jepsen et al., 2007), and in embryonic stem cells (ESCs), JMJD3 is required for neural commitment (Burgold et al., 2008). While knockdown studies in the embryonic spinal cord and retina indicate developmental roles for *Jmjd3* (Akizu et al., 2010; Iida et al., 2014), the function of JMJD3 in adult neural stem cells (NSCs) has not been reported.

The adult mammalian brain harbors NSCs in the subventricular zone (SVZ) and dentate gyrus of the hippocampus (Fuentelba et al., 2012; Ming and Song, 2011). The epigenetic mechanisms required to maintain lifelong neurogenesis in these germinal zones are only beginning to be elucidated (Gonzales-Roybal and Lim, 2013; Ma et al., 2010). Emerging evidence indicates that dynamic changes of chromatin modifications at transcriptional enhancers are a strong determinant of gene expression (Calo and Wysocka, 2013). Like promoters, the activity of enhancers can be 'poised' by the presence of repressive H3K27me3 (Rada-Iglesias et al., 2011), suggesting that transcription can be activated by the action of H3K27me3-specific demethylases at enhancer regions. Our data support a model in which adult NSCs maintain a distinct set of transcriptional regulatory elements in a poised chromatin state, and that JMJD3 can rapidly activate lineage-specific gene expression via H3K27 demethylation at specific genomic regions including enhancers.

RESULTS

JMJD3 is expressed in the adult SVZ neurogenic lineage

Throughout adult life, SVZ NSCs (type B1 cells) produce transit-amplifying cells (type C cells), which give rise to neuroblasts (type A cells) that migrate to the olfactory bulb (OB) where they become interneurons (Figure S1A and S1B). RNA-seq and *in situ* hybridization analysis revealed prominent *Jmjd3* expression in the SVZ, the neuroblast rostral migratory stream (RMS), and OB (Figure S1C and S1D, Lein et al., 2007). SVZ NSCs express glial fibrillary acidic protein (GFAP), and many (79.5%, n=30/38) GFAP+ SVZ cells exhibited nuclear JMJD3 (Figure S1E). Transit amplifying cells and neuroblasts express DLX2, and most (97.6%, n=280/287) DLX2+ cells co-expressed JMJD3 (Figure S1F and S1I). JMJD3 was also present in Doublecortin (DCX)+ neuroblasts (Figure S1G). Thus, JMJD3 is expressed SVZ NSCs as well as their neurogenic daughter cells.

JMJD3 is required for postnatal OB neurogenesis

To study the role of JMJD3 in SVZ-OB neurogenesis, we used a conditional knockout allele of *Jmjd3* (*Jmjd3^{F/F}*, Figure S2A-S2C). Mice with the *hGFAP-Cre* transgene exhibit excision of conditional alleles in SVZ NSC precursors at E13.5 (Lim et al., 2009), and SVZ cells of *hGFAP-Cre;Jmjd3^{F/F}* mice were JMJD3-negative (Figure S1J). *hGFAP-Cre;Jmjd3^{F/F}* mice and their littermate controls (wild-type and *hGFAP-Cre;Jmjd3^{F/+}*) were born at the expected Mendelian ratios and were similar in size, weight and overall survival.

To assess OB neurogenesis, we injected postnatal day 30 (P30) mice with 5-bromo-2-deoxyuridine (BrdU) to label a cohort of newly born SVZ neuroblasts and analyzed the OB for BrdU+ neurons 10 d later. In the *hGFAP-Cre;Jmjd3^{F/F}* OB, there were approximately 50% fewer BrdU+, NeuN+ neurons (Figure 1A-1C), which was not likely related to changes in neuronal survival, as the number of activated Caspase3+ OB cells was not increased (Figure 1D).

To evaluate the production of neuroblasts in the SVZ, we administered the thymidine analog ethynyl deoxyuridine (EdU) to mice 1 h before being culled. In P40 *hGFAP-Cre;Jmjd3^{F/F}* mice, there were 2-3 fold fewer DCX+, EdU+ cells in the SVZ (Figure 1H-1I). Furthermore, the expression of DLX2, a key neurogenic transcription factor, was strongly reduced (Figure 1K and 1K'). Despite there being fewer EdU+ cells in *hGFAP-Cre;Jmjd3^{F/F}* mice, the dorsal SVZ was abnormally expanded with DCX+ cells (Figure 1F-1G' and S2D-S2F'). Defective neuroblast migration can result in the postnatal accumulation of DCX+ cells in the SVZ (Lim et al., 2009); in *hGFAP-Cre;Jmjd3^{F/F}* mice, the neuroblast migratory pathways were highly disorganized (Figure S2K and S2K'), and many SVZ cells pulse-labeled with BrdU failed to migrate from the SVZ (Figure S2G-S2I'). Thus, in *hGFAP-Cre;Jmjd3^{F/F}* mice, the addition of new neurons to the OB was abrogated by a decrease in SVZ neurogenesis as well as abnormal neuroblast migration.

Adult SVZ NSCs (type B1 cells) contact the ventricle with a specialized apical surface located at the center of a pinwheel-like structure comprised of ependymal cells (Mirzadeh et al., 2008). Interestingly, adult *hGFAP-Cre;Jmjd3^{F/F}* mice had 3-4 fold more SVZ cells with such ventricular contact (Figure S2M-S2O). As is characteristic of type B1 cells, these

apical surfaces had solitary basal bodies and were GFAP⁺ (Figure S2P-S2S'). This accumulation of type B1-like cells was evident by P7 (Figure S3A-S3L') and not likely related to cell proliferation (Figure S3M-S3R'). Thus, although *hGFAP-Cre;Jmjd3^{F/F}* mice had greater numbers of cells with SVZ NSC characteristics, the production of neuroblasts was reduced, suggesting that the ventricle-contacting SVZ cells in *hGFAP-Cre;Jmjd3^{F/F}* mice are defective for neurogenesis.

***Jmjd3*-deletion targeted to adult SVZ NSCs inhibits OB neurogenesis**

To test whether *Jmjd3* plays a role in adult neurogenesis independent of its potential function in postnatal SVZ NSC development, we targeted *Jmjd3*-deletion to SVZ NSCs in adult mice (Figure 1L-1M'). Stereotactic injection the Ad:GFAP-Cre adenovirus into the SVZ induces Cre-mediated recombination in GFAP⁺ NSCs (Mirzadeh et al., 2008). We injected Ad:GFAP-Cre into the SVZ of P60-90 *Jmjd3^{F/F}* mice or littermate controls (*Jmjd3^{F/+}*) that carry the Ai14-tdTomato Cre-reporter transgene. To control for experimental variation related to the stereotactic injection itself, the Ad:GFAP-Cre adenovirus was mixed and co-injected with a lentivirus that constitutively expresses only GFP (LV-GFP).

14 d after injection, we quantified the number of tdTomato⁺ OB neurons in *Jmjd3^{F/F};Ai14* and control;*Ai14* mice (Figure 1N-1P). For each mouse, the number of tdTomato⁺ OB neurons was normalized to the number of GFP⁺ OB neurons. In control;*Ai14* mice, there were 5.5 tdTomato⁺ cells per 10 GFP⁺ cells in the OB. In contrast, there were only 2.0 tdTomato⁺ cells per 10 GFP⁺ cells in the OB of *Jmjd3^{F/F};Ai14* mice. Thus, *Jmjd3*-deleted SVZ NSCs produced 60% fewer OB neurons than NSCs in control mice. Furthermore, in the SVZ of *Jmjd3^{F/F}* mice, there were 24% fewer DLX2⁺, tdTomato⁺ cells (Figure S2T-S2W). Taken together, these results indicate that adult NSCs require *Jmjd3* for SVZ-OB neurogenesis.

***Jmjd3* regulates the differentiation of SVZ NSCs**

We next used SVZ NSCs monolayer cultures to study JMJD3 function. During differentiation, SVZ NSCs up-regulated *Jmjd3*, correlating with increased *Dlx2* expression (Figure S4A-S4G). Short-hairpin RNA (shRNA) *Jmjd3*-knockdown lentiviruses (LV-KD1-GFP and LV-KD2-GFP) strongly reduced *Jmjd3*. *Jmjd3*-knockdown reduced *Dlx2* expression, but not the expression of proneural *Mash1*, PRC2 component *Ezh2*, or *Utx* (*Kdm6a*), the other known H3K27me3-specific demethylase (Figure 2A and 2B).

To target shRNA knockdown to GFAP⁺ SVZ NSCs, we used an EnvA-pseudotyped lentivirus and tva receptor transgenic mouse strategy (Holland et al., 1998; Lewis et al., 2001). (Figure 2A). In self-renewal conditions, *Jmjd3* knockdown in GFAP⁺ SVZ NSCs did not affect BrdU incorporation or cell viability, and the expression of NSC marker *Sox2* and cell cycle inhibitor *p16* was unaffected, suggesting that NSCs self-renewal was not impaired (Figure S4H, S4I, and S4M). After 2 d of differentiation, there were approximately 50% fewer DLX2⁺ cells with *Jmjd3* knockdown, and the production of Tuj1⁺ neurons after 4 d was reduced by nearly 5-fold (Figure 2C-2G). While *Jmjd3* knockdown did not reduce the number of GFAP⁺ astrocytes, the production of OLIG2⁺ cells and O4⁺ oligodendrocytes

was impaired (Figure S4J-S4L). Thus, *Jmjd3* is required for proper SVZ NSC differentiation.

The cis-regulatory regions of *Jmjd3* harbor retinoic acid (RA) receptor response elements (Jepsen et al., 2007), and RA regulates postnatal SVZ neurogenesis *in vivo* (Wang et al., 2005). The addition of RA to SVZ NSCs increased *Jmjd3* expression by 2-3 fold, and *Dlx2* was also induced 8-9 fold (Figure 2H). Furthermore, transient transfection of a *Jmjd3* expression vector – but not a construct in which the catalytic JmjC domain is inactivated (*Jmjd3-Mut*) (Burgold et al., 2008) – increased the expression of both *Dcx* and *Dlx2* (Figure 2I). Thus, in SVZ NSCs, *Jmjd3* can be upregulated by RA, and increased levels of *Jmjd3* induces neurogenic gene expression.

***Dlx2* overexpression rescues neurogenesis from *Jmjd3*-deficient SVZ NSCs**

To investigate whether *Dlx2* is a key developmental factor for *Jmjd3*-dependent neurogenesis, we enforced *Dlx2* expression in *Jmjd3*-deficient SVZ cells. GFAPP-tva SVZ cultures were first infected with LV(EnvA)-shRNA-GFP constructs. After 2 d, cultures were infected with lentiviruses that express *Dlx2* (LV-*Dlx2*-DsRed) or control (LV-AP-DsRed) (Figure 2J). Remarkably, LV-*Dlx2*-DsRed rescued neuronal differentiation from *Jmjd3*-deficient cells (Figure 2K-2M). Furthermore, injection of LV-*Dlx2*-GFP into the SVZ *hGFAP-Cre;Jmjd3^{F/F}* mice increased the number of DCX+ cells (Figure S4N-S4R). Taken together, these results suggest that the activation of *Dlx2* is a key aspect of *Jmjd3*-dependent neurogenesis.

JMJD3 is required for H3K27me3 at key transcriptional promoters in SVZ NSCs

To gain broader insight into *Jmjd3*-dependent gene expression, we performed microarray analysis. Treatment of SVZ NSCs from UBC-Cre/ERT2;*Jmjd3^{F/F}* mice with 4-OHT resulted in *Jmjd3* deletion, and neurogenesis was severely impaired (Figure 3A, 3B, and S5A). In non-deleted cultures, 1050 genes were upregulated after 30 h of differentiation (>1.5 fold, FDR-corrected P-value < 0.05, n=3). In differentiating *Jmjd3*-deleted cells, 18 of these genes including *Dlx2* failed to reach the same levels of expression (>1.5 fold decreased as compared to control cells, FDR-corrected P-value < 0.05, n=3, Figure 3C). Analysis of ChIP-seq data (Ramos et al., 2013) indicated that 12 of the 18 genes were enriched for H3K27me3 in undifferentiated SVZ NSCs (Figure 3D), suggesting that removal of this repressive mark is required for their upregulation.

In differentiating SVZ cells, the increased expression of *Myt1*, *Slc32a1*, and *Gjb6* – genes involved in neurogenesis – was strongly *Jmjd3*-dependent (Figure 3E). In undifferentiated NSCs, these gene promoters were enriched for H3K27me3, and in control cells, H3K27me3 levels were reduced during differentiation (Figure 3F), and JMJD3 protein was detected at these promoters (Figure 3G). In contrast, in *Jmjd3*-deleted cells, H3K27me3 levels were not decreased with differentiation (Figure 3F'). Thus, JMJD3 is required for H3K27 demethylation at specific gene promoters during SVZ NSC differentiation.

The promoter region upstream of the *Dlx2* transcriptional start site (TSS) contains regulatory elements (Ghanem et al., 2012). Interestingly, in undifferentiated SVZ NSCs, the TSS and

proximal promoter was not enriched with H3K27me3 (Figure S5B). Furthermore, this low level of H3K27me3 did not change during differentiation, indicating that H3K27me3 levels at the promoter do not correlate with *Dlx2* upregulation.

JMJD3 is enriched at neural transcriptional enhancers

Some transcriptional enhancers in stem cells exist in a poised state, which includes H3K27me3 enrichment. To investigate whether JMJD3 localizes at enhancers in neural development, we analyzed JMJD3 ChIP-seq data from NSCs derived from E12.5 mouse cortex (Estarras et al., 2012) in conjunction with p300 localization in E11.5 cortex (Visel et al., 2013). Of the 4425 potential enhancer regions bound by p300, 2611 (59%) exhibited JMJD3 enrichment (Figure 4A and 4C). These JMJD3-enriched enhancers correlated with brain development (McLean et al., 2010), including differentiation toward neuronal lineages (Figure 4B). Together, these data suggest that JMJD3 can regulate the chromatin state of neural-specific enhancers genome-wide. We therefore hypothesized that JMJD3 regulates *Dlx2* expression in SVZ NSCs through interactions at a key enhancer.

JMJD3 is required for H3K27me3 demethylation of a *Dlx2* enhancer

I12b is an enhancer that regulates the expression of *Dlx2* (Poitras et al., 2007) (Figure 5A). In SVZ NSC cultures, I12b had a poised chromatin state, which included high levels of H3K27me3 (Figure S5C and S5D). To analyze I12b chromatin state changes in the SVZ neurogenic lineage, we used fluorescent activated cell sorting (FACS) to isolate GFAP+, NESTIN+ SVZ NSCs in self-renewal conditions and Tuj1+ neuroblasts 3 d after differentiation (Figure 5B-5E). In NSCs, I12b was enriched with H3K27me3. In the neuroblasts, H3K27me3 was decreased ~12 fold (Figure 5C). This reduction in H3K27me3 correlated with JMJD3 enrichment at I12b (Figure 5D). Furthermore, in neuroblasts, H3K27 acetylation (H3K27ac) was increased (Figure 5E). Thus, JMJD3 enrichment at I12b correlated with the chromatin state activation of this *Dlx2* enhancer. Another *Dlx1/2* enhancer, URE2, had lower levels of H3K27me3 that did not decrease during neuronal differentiation (Figure S5C and S5E). We therefore focused our analysis upon I12b.

We next studied SVZ NSCs with and without acute *Jmjd3*-deletion. During differentiation, H3K27me3 at I12b was decreased in control cells. In contrast, in *Jmjd3*-deleted cells, H3K27me3 did not decrease at the I12b enhancer (Figure 5F and 5F'), correlating with the lack of *Dlx2* upregulation (Figure 3C). Furthermore, JMJD3 was enriched at I12b during differentiation (Figure 5G). Thus, *Jmjd3* is required for H3K27 demethylation at the I12b enhancer.

In differentiating *Mll1*-deficient SVZ cells, the *Dlx2* locus is enriched with H3K27me3 and its transcription remains repressed (Lim et al., 2009). We therefore investigated a potential molecular-genetic relationship between *Mll1* and *Jmjd3* in the regulation of *Dlx2*. In differentiating SVZ cells, MLL1 was localized at both I12b and the *Dlx2* promoter (Figure 5H). Interestingly, in *Mll1*-deleted NSCs, JMJD3 was not enriched at I12b (Figure 5I) and *Dlx2* is not properly expressed. However, in *Jmjd3*-deleted cells, MLL1 remained enriched at I12b (Figure 5J). Taken together, these data indicate that *Mll1* is required for the enrichment of JMJD3 at I12b.

DISCUSSION

Our study demonstrates that JMJD3 is required for the life-long neurogenesis of SVZ NSCs and further illustrates mechanisms by which JMJD3 activates neurogenic gene expression. In addition to acting at gene promoters, JMJD3 also localizes to neural transcriptional enhancers and regulates the chromatin state of the I12b enhancer of *Dlx2* (Figure S5F).

While multiple studies have indicated the critical nature of Polycomb-mediated transcriptional repression in neural development (Hirabayashi and Gotoh, 2010; Hwang et al., 2014), the role of active H3K27me3 demethylation has been less clear. How do H3K27me3-repressed genes normally become activated? While loss of H3K27me3 at specific loci could potentially be passive (e.g., through decreased EZH2 activity), EZH2 expression is not downregulated during SVZ neurogenesis (Hwang et al., 2014). In this study, we show that active H3K27me3 demethylation via JMJD3 is required for efficient expression of lineage-specific genes.

Without *Jmjd3* SVZ NSCs appear to be “stalled” in a precursor cell state. *Jmjd3*-deficient SVZ NSCs did not efficiently activate *Dlx2* expression and were defective for neurogenesis. However, *Jmjd3* did not appear to be required for NSC identity, suggesting that *Jmjd3* does not play a key role in SVZ NSC maintenance. Interestingly, adult *hGFAP-Cre;Jmjd3^{F/F}* mice had greater numbers of cells with type B1 cell characteristics. Given that we did not observe increased cell proliferation with *Jmjd3*-deficiency, it is possible that the accumulation of type B1-like cells in *hGFAP-Cre;Jmjd3^{F/F}* mice relates to an increase in the proportion of symmetric, self-renewing divisions, as compared to asymmetric NSC divisions that lead to the generation of differentiated cell types.

In the developing neocortex (Estaras et al., 2012; Visel et al., 2013), we found that 59% of the mapped enhancers exhibited JMJD3 enrichment. Notably, these enhancers corresponded strongly to neuron differentiation, forebrain development, regulation of neurogenesis, and other activities consistent with NSC differentiation and cortical development. These data support a role for JMJD3 at neural enhancers genome-wide in neural precursor cells, including those in the developing brain.

The requirement of JMJD3 for H3K27me3 demethylation at the I12b enhancer does not diminish the function of JMJD3 at gene promoter regions. Indeed, JMJD3 enrichment has been mapped to 7170 promoter regions in NSCs cultured from E12.5 cortex (Estaras et al., 2012), and JMJD3-dependent H3K27me3 demethylation occurs at a number of different neural gene promoters (Akizu et al., 2010; Burgold et al., 2008; Iida et al., 2014). In this study, we found that the promoter regions of *Myt1*, *Slc32a1*, and *Gjb6* required JMJD3 for H3K27me3-demethylation and transcriptional activation. Taken together, these data suggest that JMJD3 acts at both promoters and enhancers in NSC populations.

While UTX (KDM6A) – the other known H3K27me3-specific demethylase (Lan et al., 2007) – is required to activate gene expression in the developing heart, *Utx*-null ESCs can efficiently differentiate into neurons, suggesting that UTX is not necessary for neuronal differentiation *per se* (Lee et al., 2012). In SVZ NSCs, *Jmjd3*-deficiency did not affect the expression of *Utx*, indicating that *Utx* alone is not sufficient to activate *Dlx2* expression, and

RNAi knockdown of *Utx* in SVZ cells did not inhibit neurogenesis (data not shown). Thus, different developmental lineages may utilize UTX and JMJD3 in a non-redundant manner.

How JMJD3 becomes localized at specific genomic regions is not well known, but emerging evidence indicates that transcription factors such as SMAD3 and HES1 are involved (Dai et al., 2010; Estaras et al., 2012). JMJD3 has also been found in trithorax group (trxG) chromatin remodeling complexes (De Santa et al., 2007), and trxG family member *Mll1* is required for SVZ neurogenesis (Lim et al., 2009). In SVZ cells, we found both MLL1 and JMJD3 proteins localized at I12b, and JMJD3 enrichment at this enhancer was lost in *Mll1*-deleted cells. While these data indicate that JMJD3 requires MLL1 for localization to a key neurogenic enhancer, it remains to be determined whether MLL1 and JMJD3 physically interact at I12b. Alternatively, MLL1 may be required to mediate local chromatin modifications that enable the subsequent recruitment of JMJD3 to specific genomic regions. The discovery of this genetic interaction may provide new insights into how mutations in MLL genes and H3K27me₃-specific demethylases contribute to the transcriptional dysregulation of medulloblastomas (Jones et al., 2012; Parsons et al., 2011). Finally, our studies establish JMJD3 as a central player for the activation of neurogenesis from a neural stem cell population, which may help inform methods of neuronal production for therapeutic purposes.

EXPERIMENTAL PROCEDURES

Generation of *Jmjd3* conditional knockout mice and mouse studies

Jmjd3^{F/F} mice were generated, maintained and genotyped as described (Iwamori et al., 2013). BrdU (50 µg/gram body weight, Sigma) or EdU (10⁻²µmol/gram body weight, Invitrogen) were injected intraperitoneally. Stereotactic SVZ injections were performed essentially as described (Mirzadeh et al., 2008). Briefly, for adult *Jmjd3*-deletion studies, we co-injected 100nl of adenovirus (Ad:GFAP-Cre) and LV-GFP lentivirus. For *Dlx2* overexpression, we co-injected 100nl of LV-*Dlx2*-GFP and LV-*AP*-DsRed into the SVZ. Stereotactic SVZ coordinates: 0.5 mm anterior, 1.3 mm lateral (relative to bregma), 1.75 mm deep to the pia. Brains were fixed by intracardiac perfusion and sectioned on a cryostat (Leica) at 12 or 16-µm thickness. After blocking, primary antibodies were incubated at 4 °C overnight. Antibody dilutions are listed in the Supplemental Experimental Procedures. Whole-mount dissection and immunostaining was performed as described (Mirzadeh et al., 2008). From each animal, we analyzed at least 3 separate tissue sections, and from each section, we collected > 3 non-overlapping confocal images (Leica TCS SP5X) with 20X or 63X-oil objectives for quantification (ImageJ, NIH). Experiments were performed in accordance to protocols approved by Institutional Animal Care and Use Committee at UCSF.

Cell culture studies

SVZ NSC monolayer cultures were generated and analyzed as in (Lim et al., 2009). High-titer pSicoR lentiviruses were produced in HEK 293T cells as described in (Lewis et al., 2001). For *Jmjd3*-knockdown, we derived SVZ NSCs from transgenic mice that express the *tva* receptor under the control of the GFAP promoter (GFAPP-*tva*) (Holland et al., 1998). To

delete *Jmjd3* in SVZ NSCs, cultures from UBC-Cre/ERT2;*Jmjd3*^{F/F} mice were treated with 4-OHT (50nM, Sigma) for 96 h. For retinoic acid (RA) treatment and *Jmjd3* overexpression, SVZ NSC monolayer cultures were established as described in (Brill et al., 2008). For *Jmjd3* overexpression, pCDNA-Flag *Jmjd3* and pCDNA-Flag *Jmjd3* mutant (H1388/A) (Burgold et al., 2008) were transfected using jetPRIME (Polyplus) and analyzed after 48 hrs. For each culture well, we captured > 13 thirteen non-overlapping fields Leica DMI4000B) with a 20X objective and analyzed with ImageJ (NIH). Primary and secondary antibodies are in the Supplemental Experimental Procedures. SYTOX Red (Invitrogen) was used per manufacturer's protocol and quantified on a fluorescent flow cytometer (BD FACSAria).

ChIP, RT-qPCR, microarray, and bioinformatic analysis

qChIP and RT-qPCR was performed essentially as previously described (Lim et al., 2009). qChIP analysis of FACS isolated cells was performed as in (Hwang et al., 2014). ChIP antibodies and primer sequences and other methodological details are in Supplementary Information. For microarray analysis, biological replicates were prepared as in (Ramos et al., 2013) and hybridized to MouseRef-8 v2.0 Expression BeadChip arrays (Illumina). Array data were normalized with IlluminaNormalizer v.2 and analyzed with Cyber-T (<http://cybert.ics.uci.edu/>). Gene Ontology (GO) analysis was performed with DAVID. SVZ RNA-seq and SVZ NSC ChIP-seq data was analyzed as in (Ramos et al., 2013). ChIP-seq data from GEO series GSE36673, GSE42881, and GSE13845 were analyzed as follows: FASTQ reads were aligned to mm9 using Bowtie2 2.1.0 with default parameters. Tracks and peaks were generated using MACS 1.4 with default parameters. Co-occupancy analysis was performed using BEDTools against published peaks. Genomic Regions Enrichment of Annotations Tool (GREAT) analysis was performed as in (McLean et al., 2010). Analysis parameters and references are in Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by VA 1101 BX000252-01, NIH DP2-OD006505-01, and the Sontag Foundation to D.A.L., and by the Italian Ministry of Health and Italian Association for Cancer Research (AIRC) to G.T. We thank Drs. Arturo Alvarez-Buylla and John Rubenstein for insightful comments on this work and the manuscript.

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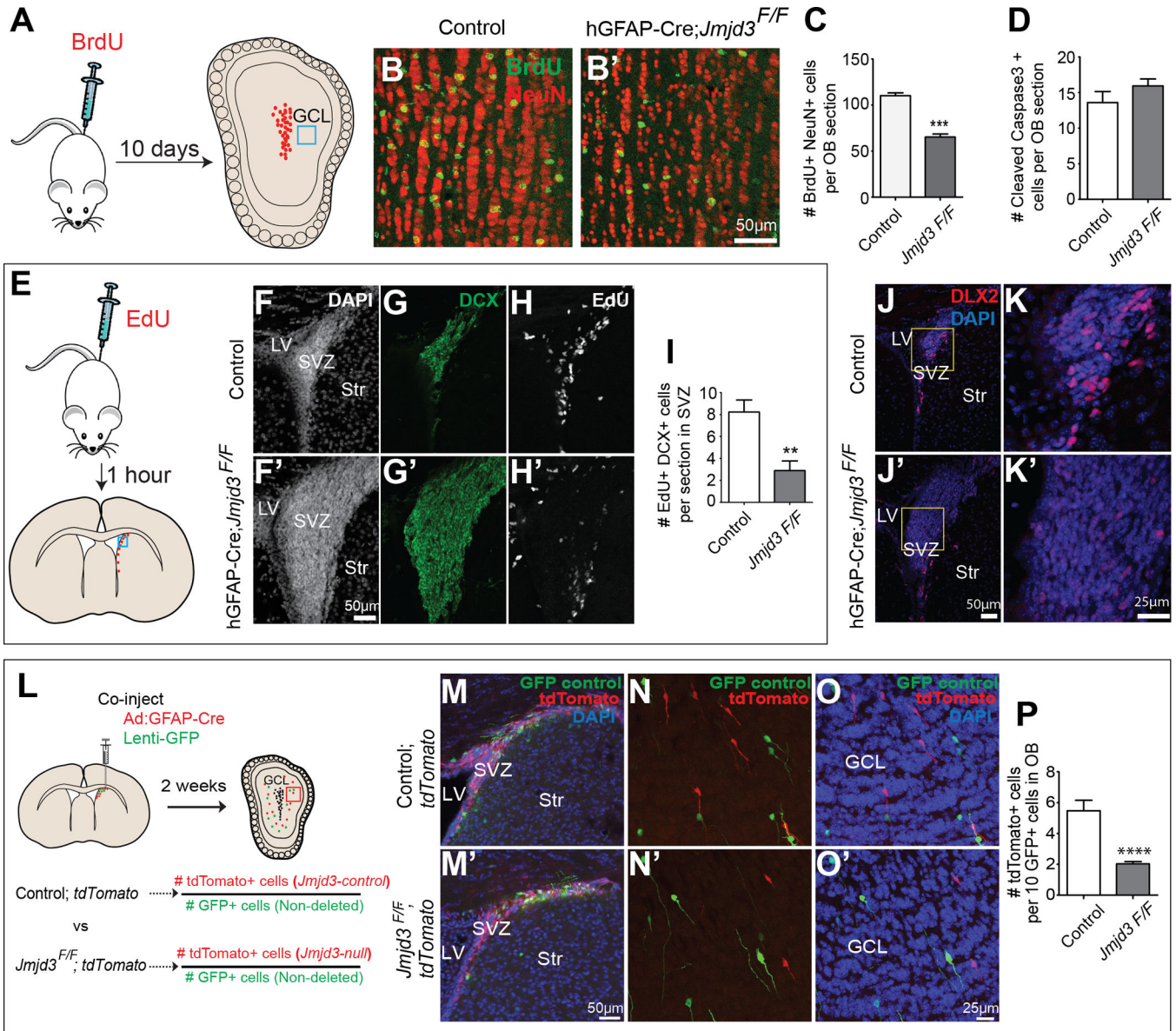


Figure 1. *Jmjd3* is required for adult OB neurogenesis

(A) Illustration of the experimental design for B-D. Green box indicates regions of shown in B and B'. GCL, granule cell layer. (B-D) Analysis of OB neurogenesis. (B and B')

Immunohistochemistry (IHC) for BrdU (green) and NeuN (red) in coronal OB sections of control (B) and *hGFAP-Cre;Jmjd3^{F/F}* mice (B'). (C) Quantification of BrdU+, NeuN+ OB neurons (***) $P < 0.001$, $n = 4$ each, error bars, s.e.m.). (D) Quantification of Caspase3+ OB cells ($P = 0.1933$, $n = 4$ each, error bars, s.e.m.)

(E) Illustration of the experimental design for F-I. EdU was injected 1h before analysis. (F-I) Analysis of cell proliferation and neuroblasts in the SVZ. (F-H) DAPI+ (white), DCX+ (green), and EdU+ (white) cells in the SVZ of control mice. (F'-H') DAPI+ (white), DCX+ (green), and EdU+ (white) cells in the SVZ of *hGFAP-Cre;Jmjd3^{F/F}* mice. (I) Quantification of EdU+, DCX+ cells in SVZ coronal sections ($n = 3$ each, error bars, s.e.m., ** $P < 0.01$).

(J-K') Analysis of DLX2 expression in the SVZ. IHC for DLX2 (red) in SVZ coronal sections of control (J-K) and *hGFAP-Cre;Jmjd3^{F/F}* mice (J'-K'). Panels K and K' are higher magnification views of the yellow boxed region in J and J'.

(L) Schematic illustration of the experimental design for M-P. Ad:GFAP-Cre virus (to delete floxed alleles in GFAP+ SVZ NSCs) and GFP lentivirus (injection control) were co-injected into the adult SVZ of *tdTomato;Jmjd3^{F+}* (M-O) or *tdTomato;Jmjd3^{FF}* (M'-O') mice. (M-P) Analysis of OB neurogenesis. (M and M') IHC for GFP (green) and tdTomato (red) in adult SVZ coronal sections of *tdTomato;Jmjd3^{F+}* (M) and *tdTomato;Jmjd3^{FF}* mice (M'). (N-O') IHC for GFP (green) and tdTomato (red) in coronal OB sections of *tdTomato;Jmjd3^{F+}* (N and O) and *tdTomato;Jmjd3^{FF}* mice (N' and O') 14 d after injection. (P) Quantification of tdTomato+ neurons per 10 GFP+ neurons in OB (**** $P < 0.0001$; n=4 per group; error bars, s.e.m.). LV, lateral ventricle; Str, Striatum; GCL, granule cell layer.

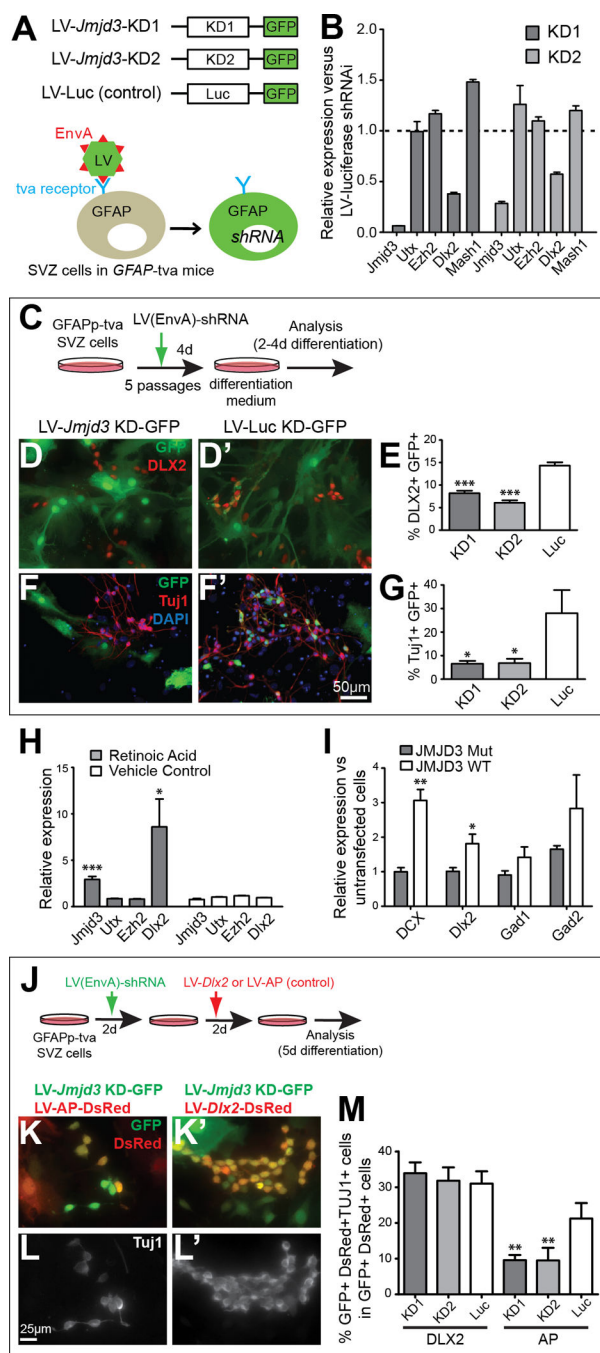


Figure 2. *Jmjd3* regulates SVZ NSC differentiation

(A) EnvA and *hGFAP-tva* strategy of targeting knockdown to GFAP+ SVZ NSCs. EnvA (red) pseudotyped lentiviruses can only infect cells with the *tva* receptor (blue), which is expressed from the *GFAP-tva* transgene. (B) RT-qPCR analysis of LV-*Jmjd3* knockdown in SVZ NSCs (error bars, s.e.m.).

(C) Illustration of the experimental design for D-G. (D-E) ICC for GFP (green) and DLX2 (red) with LV-sh-*Jmjd3* (D) and LV-sh-luciferase (D'). (E) Quantification of DLX2+ cells. (F-G) ICC for GFP (green) and Tuj1 (red) with LV-sh-*Jmjd3* (F) and LV-sh-luciferase (F').

- (G) Quantification of Tuj1+ cells (error bars, s.e.m. of quadruplicate cultures, $*P<0.05$, $**P<0.01$).
- (H) RT-qPCR analysis of retinoic acid treatment of SVZ NSCs ($***P<0.001$, $*P<0.05$; n=6 per group; error bars, s.e.m).
- (I) RT-qPCR analysis of *Jmjd3* overexpression in SVZ NSCs. ($**P<0.01$. $*P<0.05$; n=3; error bars, s.e.m).
- (J) Illustration of the experimental design for K-M. (K-M) Analysis of neuronal differentiation in *Jmjd3*-deficient cells after *Dlx2* overexpression. (K-L') IHC for Tuj1 (white) in *Jmjd3*-deficient cells with *Dlx2* overexpression (yellow in K', corresponding white cells in L') and control vector expression (yellow in K, corresponding white cells in L). (M) Quantification of *Dlx2* rescue of neurogenesis (error bars, s.e.m. of quadruplicate cultures, $**P<0.01$, $***P<0.001$).

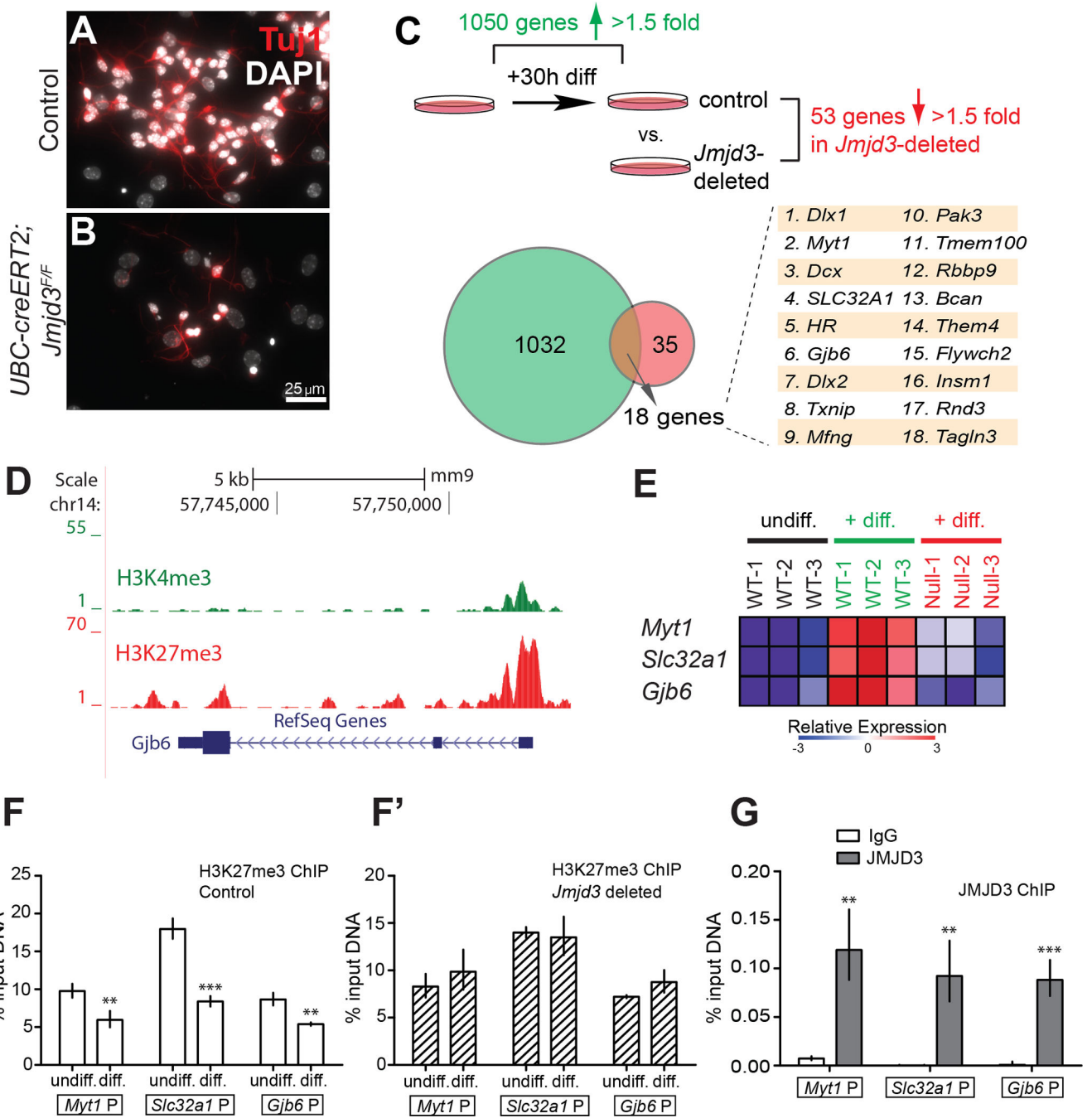


Figure 3. JMJD3 is required for H3K27me3 demethylation at neural gene promoters in SVZ NSCs

(A and B) ICC for Tuj1 (red) in SVZ NSC cultures from P8 *UBC-Cre/ERT2;Jmjd3^{F/F}* mice after 4 d of differentiation. *Jmjd3* was deleted in SVZ NSCs by 50 μ m 4-OHT treatment. (C) Top, schematic of microarray analysis of SVZ cell differentiation and differential expression between *Jmjd3*-deleted and control cells. Bottom left, Venn diagram depicting overlap between normal gene upregulation and *Jmjd3*-dependent upregulation; 18 genes in the overlap are listed. (D) H3K4me3 and H3K27me3 ChIP-seq enrichment tracks at the *Gjb6*

locus from undifferentiated NSC cultures. H3K27me3 is enriched at the *Gjb6* promoter located at the right. (E) Heatmap of gene expression profile for *Myt1*, *Slc32a1*, *Gjb6*. (F-F') qChIP analysis of H3K27me3 in control (EtOH) and *Jmjd3*-deleted cells (4OHT) at *Myt1*, *Slc32a1*, and *Gjb6* promoters. White bars correspond to control cells. Hatched bars correspond to *Jmjd3*-deleted cells. *** $P < 0.001$. ** $P < 0.01$, compared to proliferating cells. (G) qChIP analysis of JMJD3 at *Myt1*, *Slc32a1*, and *Gjb6* promoters in differentiating NSCs. *** $P < 0.001$. ** $P < 0.01$, compared to IgG. Error bars, s.d., n=3. undiff = undifferentiated SVZ NSCs, +diff = differentiated SVZ cells.

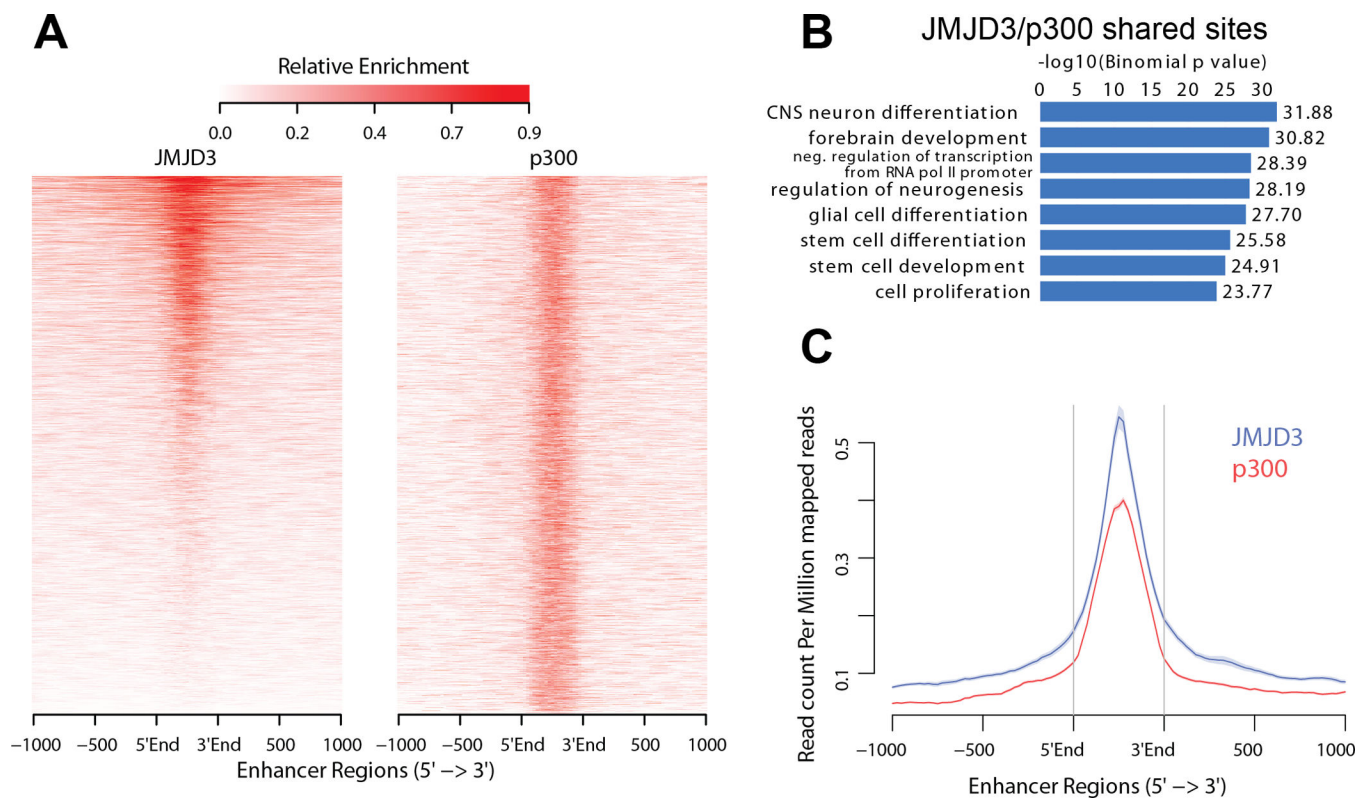


Figure 4. JMJD3 is enriched at neural transcriptional enhancers

(A) JMJD3 and p300 enrichment across 4425 neural enhancers and their flanking regions.

(B) The top 8 enriched gene ontology terms identified using GREAT analysis for the 2611

JMJD3 and p300 enriched enhancers. (C) Averaged enrichment profile of JMJD3 and p300 across 4425 putative neural enhancers and their flanking regions.

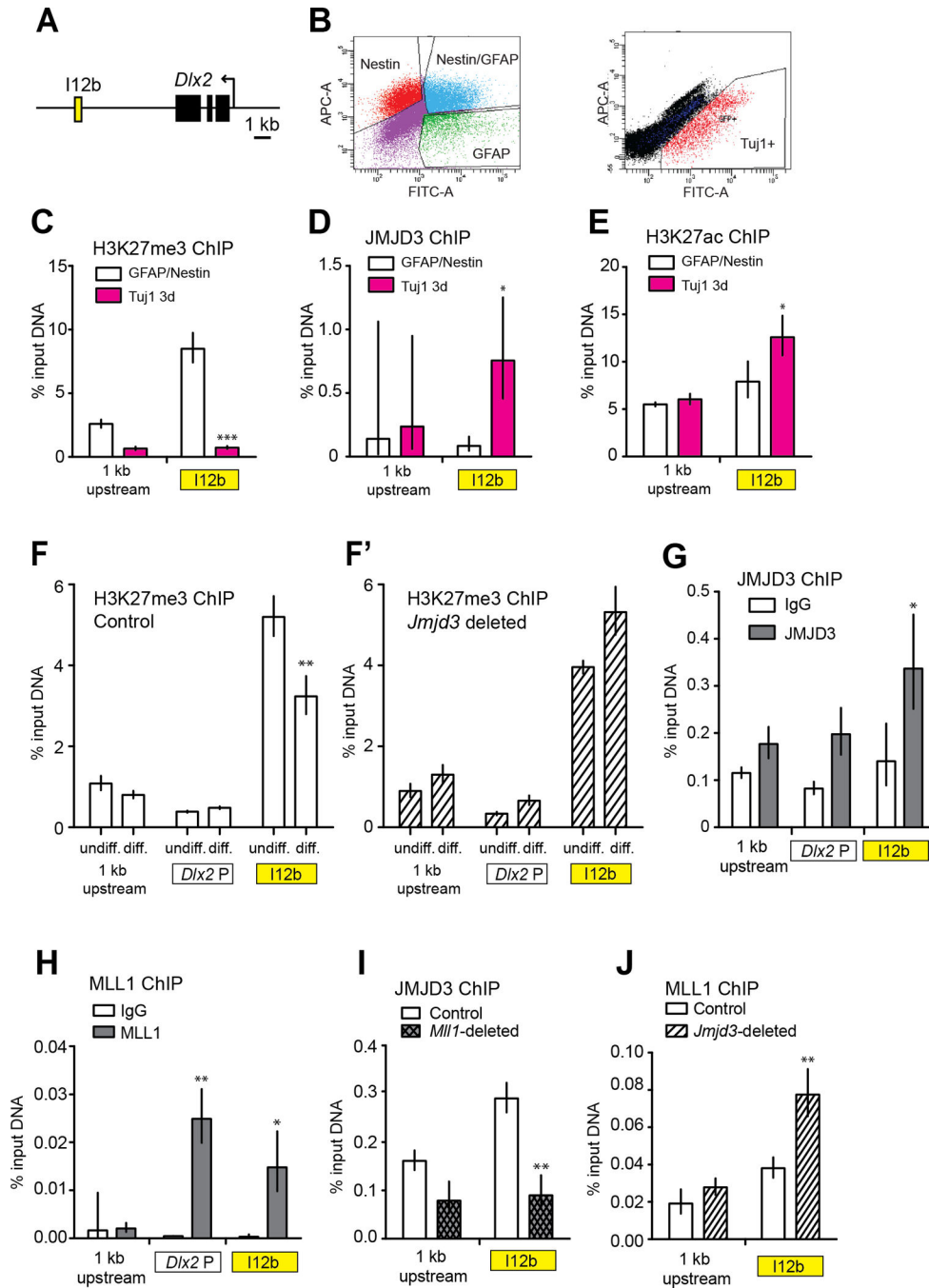


Figure 5. JMJD3 is required for the activation of the I12b enhancer

(A) The genomic region including *Dlx2* and the I12b enhancer (yellow). Arrow indicates the *Dlx2* promoter. A region 1kb upstream of *Dlx2* was also analyzed. (B) FACS plots of SVZ cell isolation. (C-E) qChIP analysis of H3K27me3 (C) JMJD3 (D) and H3K27ac (E) in FACS isolated GFAP⁺, NESTIN⁺ cells and TUJ1⁺ cells. ****P*<0.001. **P*<0.05. (F-F') qChIP analysis of H3K27me3 in control (EtOH) and *Jmjd3*-deleted cells (4OHT) at the I12b enhancer and *Dlx2* promoter. White bars correspond to control cells. Hatched bars correspond to *Jmjd3*-deleted cells. ***P*<0.01, compared to proliferating control cells.

(G) qChIP analysis of JMJD3 at I12b and the *Dlx2* promoter in differentiating NSCs. * $P < 0.05$, compared to 1kb upstream region.

(H) qChIP analysis of MLL1 at *Dlx2* promoter and I12b enhancer during NSC differentiation. ** $P < 0.01$, * $P < 0.05$, compared to 1kb upstream region. (I) qChIP analysis of JMJD3 at I12b during differentiation in control and *Mll1*-deleted cells. ** $P < 0.01$. (J) qChIP analysis of MLL1 at I12b during differentiation in control and *Jmjd3*-deleted cells. ** $P < 0.01$. Error bars, s.d., n=3. undiff = undifferentiated SVZ NSCs, +diff = differentiated SVZ cells.