The H3K27 Histone Demethylase Kdm6b (Jmjd3) is Induced by Neuronal Activity and Contributes to Neuronal Survival and Differentiation

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Neurobiology in the Graduate School of Duke University

2012
ABSTRACT

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Abstract

The proper development and function of neurons requires the regulated expression of specific gene programs in response to developmental and environmental cues. One way that gene expression is regulated is via post-translational modification of the N-terminal tails of the histone proteins that are associated with regulated genes. A growing body of evidence suggests that stimulus-driven modulation of histone modifications play an important role in the regulation of neuronal activity-regulated gene transcription. However, the histone modifying enzymes that are targets of activity-regulated signaling cascades in neurons remain to be identified. The histone demethylases (HDMs) are a large family of enzymes that have selective catalytic activity against specific sites of histone methylation. To identify HDMs that may be important for activity-regulated gene transcription in neurons, we induced seizures in mice and screened for HDMs whose expression is induced in the hippocampus. Among the few HDMs that changed expression, Kdm6b showed the highest induction. Kdm6b is a histone H3K27-specific HDM whose enzymatic activity leads to transcriptionally permissive chromatin environments. In situ hybridization analysis revealed that Kdm6b is highly induced in post-mitotic neurons of the dentate gyrus region of the hippocampus. We can recapitulate the activity-dependent induction of Kdm6b expression in cultured hippocampal neurons by a
stimulus that activates synaptic NMDA receptors. *Kdm6b* expression is also induced following application of brain derived neurotrophic factor (BDNF), a neurotrophic factor that is upregulated in the seized hippocampus.

To investigate possible functions of Kdm6b in neuronal development, we performed *in situ* hybridization analysis that allows for the identification of regions with high *Kdm6b* expression that could be sites of potential function in the developing mouse brain. We found high levels of *Kdm6b* expression in the inner layer of the external granule layer of the cerebellum, a region where pre-migratory immature neurons reside and a site of significant apoptosis. On the basis of this data and the fact that intracellular calcium signaling arising from synaptic firing supports neuronal survival, we explored the necessity for Kdm6b in the survival of cultured cerebellar granule neurons. Knockdown of *Kdm6b* by RNAi increases cell death, demonstrating that Kdm6b contributes to neuronal survival. On the basis of our *in situ* analysis, that shows the expression of *Kdm6b* in differentiating CGNs and the fact that Kdm6b is implicated in the differentiation of macrophages we explored the necessity for *Kdm6b* in the regulation of genes important for cerebellar granule neuron differentiation. Knockdown of *Kdm6b* by RNAi decreased the developmental upregulation of several genes that belong to a late differentiation program. Interestingly, while we did not find that *Kdm6b* is required for BDNF-mediated cerebellar granule neuron survival, our data show that *Kdm6b* is permissive for BDNF-induced
upregulation of late differentiation genes, suggesting that Kdm6b may be required for other BDNF-dependent processes in the cerebellum, such as CGN differentiation and synapse maturation. Overall these data raise the possibility that stimulus-dependent regulation of Kdm6b, and perhaps regulation of H3K27 methylation mediated by Kdm6b, may contribute to the regulation of gene expression in neurons and thus to their proper development and plasticity.
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1. Introduction

1.1 Epigenetic mechanisms of gene regulation

1.1.1 Chromatin

Eukaryotic genomes are organized into chromatin, a heterogeneous, higher order structure of DNA and protein. The basic building blocks of chromatin are nucleosomes, each of which is composed of 146bp of DNA wrapped around a histone octamer consisting of two copies each of the core histones H2A, H2B, H3, and H4 (Kouzarides, 2007). Covalent histone modifications, histone variants with different primary sequences, and chromatin remodeling complexes work together to alter the structure of the chromatin fiber (Strahl and Allis, 2000).

1.1.2 Epigenetics

The general concept of an “epigenetic landscape” was first articulated by the developmental biologist Conrad Waddington, who used it to explain how identical genotypes could yield a wide collection of phenotypes (Jaenisch and Bird, 2003). In the molecular age, the term “epigenetics” has come to encompass a specific set of molecular mechanisms that regulate the structure and function of chromatin: consistent with the original term, these modifications are “epi,” meaning layered over, “genetic,” referring to the actual DNA sequence.

Epigenetic control is largely accomplished via covalent modifications of histones and DNA, which impact transcription via the recruitment of effector mechanisms involving proteins with recognition domains specific for modified
residues (Jenuwein and Allis, 2001; Kouzarides, 2007). DNA is methylated at CpG dinucleotides, and the N-terminal tails of histones are post-translationally modified in various ways including acetylation and methylation, as described in greater detail below.

Finally, the word “epigenetic” has historically been tightly associated with the idea of heritable traits. However, we will be discussing these mechanisms of chromatin regulation in post-mitotic neurons where the idea of heritability does not apply. As background for this idea, during the development of multicellular organisms, a single cell gives rise to genetically homogenous but structurally and functionally heterogenous populations of cells via the differential expression of genes. Many of these differences in gene expression arise during development and are retained through cell divisions. DNA methylation and histone modifications are crucial for the establishment and persistence of cell-type-specific gene expression patterns through cell divisions, and are thus “heritable” modifications of chromatin. However, outside of development, molecular mechanisms we categorize as “epigenetic” may not always fit into the strict definition of epigenetics; they may not be long-lasting or heritable. Many modifications play roles in many dynamic processes such as transcriptional induction and DNA repair. In post-mitotic cells, like differentiated neurons, heritability does not apply, and epigenetic applies to long-lasting changes. In such cases, these mechanisms are epigenetic only in the sense that they
facilitate differential expression without changes in the underlying DNA sequence.

1.1.3 Histone modifications

Histones are subject to a variety of posttranslational modifications including acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation, the most recently identified crotonylation, etc. each of which occurs at specific amino acid residues of the flexible N-terminal tails of the histone proteins (Kouzarides, 2007; Tan et al., 2011) (Table1).

1.1.4 Histone modifications and gene expression

Histones are not merely structural components of chromatin; modifications on histones can affect the transcriptional state of the associated genes (Jenuwein and Allis, 2001; Kouzarides, 2007). Analysis of the correlation between histone modifications and the activation state of transcription led to the formation of the histone code hypothesis, which states that the modifications on histones associated with a specific gene determine the likelihood that the gene will be transcribed. Modifications can be divided into those that correlate with activation and those that correlate with repression. Lysine acetylation is found in the regulatory regions of transcriptionally active genes and correlates with increased transcriptional activity. Acetylation of a lysine on a histone tail
# Table 1: Histone modifications

<table>
<thead>
<tr>
<th></th>
<th>Acetylation</th>
<th>Methylation</th>
<th>Phosphorylation</th>
<th>Ubiquitination</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H2A</strong></td>
<td>K5, K9, K13, K15, K36</td>
<td>K99, K119</td>
<td>S1, T120</td>
<td>K119</td>
</tr>
<tr>
<td><strong>H2B</strong></td>
<td>K5, K12, K15, K20, K85, K108, K116, K120</td>
<td>K5, K43</td>
<td>S14</td>
<td>K20, K120</td>
</tr>
<tr>
<td><strong>H3</strong></td>
<td>K4, K9, K14, K18, K23, K27, K36, K56</td>
<td>R2, K4, R8, K9, R17, K23, R26, K27, K36, K37, K56, K79</td>
<td>T3, S10, T11, S28, Y41, T45</td>
<td></td>
</tr>
<tr>
<td><strong>H4</strong></td>
<td>K5, K8, K12, K16, K20, K77, K79, K91</td>
<td>R3, K12, K20, K59, R92</td>
<td>S1, H18, S47</td>
<td></td>
</tr>
</tbody>
</table>
removes the lysine’s positive charge, which neutralizes its interaction with the negatively charged phosphate of the DNA backbone. This relaxes the chromatin structure, thereby increasing the availability of the gene for transcription.

The relationships between the other histone modifications and gene transcription are more complex. Whether a methyl mark is repressive or active depends on the specific residue modified. Methylation of a lysine residue does not change the charge on the residue and does not introduce large alterations in chromatin structure. Rather, these modifications create platforms for the recruitment of additional regulatory proteins (Jenuwein and Allis, 2001; Schreiber and Bernstein, 2002). Domains have been identified in proteins that can bind specific methylated lysine residues, e.g. chromo- and PHD-domains. High levels of H3K4, H3K36, and H3K79 methylation are associated with gene transcription, while high levels of H3K9, H3K27, and H4K20 methylation correlate with gene repression. For example, while high H3K4 methylation is detected at the promoter regions of active genes (Bernstein and Kellis, 2005; Roh et al., 2006; Roh et al., 2005), high levels of H3K27 methylation in promoter regions correlate with gene repression (Roh et al., 2006). Furthermore, the degree of methylation also affects the association with gene expression. While di- and tri-methylation at H3K27 (H3K27me2 and H3K27me3) are associated with repression, mono-methylation at H3K27 (H3K27me) is associated with actively transcribed regions. In an example from human T cells, silent promoters have higher levels of
H3K27me2/3 compared to active promoters, while the reverse is true for H3K27me1 (Barski et al., 2007). Each of these modifications alone can affect chromatin architecture, but it is likely that in most cases a combination of these modifications determines the chromatin state, which in turn governs gene transcription.

1.1.5 PcG repression/H3K27me3 (in development)

Repression of developmental genes through histone methylation is best illustrated by Polycomb group (PcG) proteins (Ringrose and Paro, 2007). The hallmark of Polycomb repression is H3K27 methylation catalyzed by the histone methyltransferase Enhancer of Zeste Homolog 2 (Ezh2), a subunit of the Polycomb repressor complex 2 (PRC2) (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Schuettengruber et al., 2007). This mark is recognized and stabilized by PRC1 (Cao et al., 2002; Cao and Zhang, 2004; Czermin et al., 2002; Kuzmichev et al., 2002; Schuettengruber et al., 2007). Methylation of lysine 27 on histone H3 by the PcG proteins is associated with gene silencing in many developmental processes. PcG proteins are best known for their developmentally-relevant role as Hox gene repressors in regulating body patterning (Boyer et al., 2006; Lee et al., 2012). PcG repression is also important for mammalian X chromosome inactivation, imprinting, germline development, stem cell identity, cell-cycle regulation, and cancer (Bernstein et al., 2006;
Mikkelsen et al., 2007; Ringrose and Paro, 2007; Schuettengruber et al., 2007; Valk-Lingbeek et al., 2004).

1.1.6 Bivalent domains

The existence of bivalent domains challenges the simplistic view of activating vs. silencing histone modifications. Genome-wide analysis of histone lysine methylation has shown that in ES (embryonic stem) cells, promoters of many cell-type-specific genes that are largely silent carry an active H3K4me3 methyl mark in addition to the repressive H3K27me3 methyl mark (Bernstein et al., 2006; Mikkelsen et al., 2007). This combination of histone modifications is referred to as a “bivalent” domain, and it is surmised that these marks keep genes poised for rapid activation or stable inactivation. During differentiation, these bivalent domains get resolved into either the H3K4me3 mark or the H3K27me3 mark (with the other mark removed) to turn the expression of these genes either on or off, respectively. For example, in ES cells, Hox genes carry bivalent domains on their promoters and are kept silent. The loss of H3K27me3 is important for proper Hox gene expression during development (Agger et al., 2007). These data suggest that enzymes with the ability to remove H3K27me3 methyl marks are crucial for the process of cell fate determination and differentiation.

Bivalent domains are not limited to ES cells; this colocalization of opposing histone modifications has also been reported in differentiated T cells
and neural precursors (Mohn et al., 2008; Roh et al., 2006). In differentiated cells, bivalent domains may have a more complex function in fine-tuning gene expression, as suggested by a genome-wide analysis in T lymphocytes (Roh et al., 2006): genes bearing H3K4me3 could be clustered according to their H3K27me3 levels in genes with low transcriptional activity (high H3K27me3), genes with high transcriptional activity (low H3K27me3), and finally genes with intermediate levels of H3K27me3 and intermediate degrees of activity.

1.1.7 Histone demethylases (HDMs)

A steady-state level of histone methylation is the result of activities between two opposing classes of histone modifying enzymes – histone methyl transferases (HMTs) that add methyl groups and histone demethylases (HDMs) that remove methyl groups. HMTs were identified some time ago, but HDMs eluded researchers until recently. Methylation of histones is a thermodynamically very stable modification (Lachner and Jenuwein, 2002; Zhang and Reinberg, 2001). For this reason, methylation was thought of as a truly epigenetic mark before the identification of HDMs, because once a methyl mark is added it was difficult to remove (Bannister and Kouzarides, 2011; Jenuwein and Allis, 2001). It was speculated that histone methylation, by introducing stable long-lasting changes to genes, could explain such behavioral phenomena as drug addiction, sensitization, memory, and learning. However, the rapid decrease of H3K27me3 during differentiation suggested that enzymes that mediate demethylation may
exist. The controversy associated with the existence of HDMs was finally put to rest with the identification of the first HDM, Kdm1, in 2004 (Shi et al., 2004). It also brought to light the notion that histone methylation can be dynamically regulated (De Santa et al., 2007; Klose et al., 2006; Shi and Whetstine, 2007).

1.1.7.1 Kdm1/Lsd1 - the first histone demethylase

Kdm1 or Lysine-Specific Demethylase 1 (LSD1) was the first HDM to be identified (Metzger et al., 2010; Shi et al., 2004). Kdm1 does not directly cleave the N-CH3 bond from the lysine side chain to the attached methyl group. Instead, it induces amine oxidation of the methylated H4K4 to generate an unmodified lysine (formaldehyde is generated as a by product). This reaction needs the co-factor FAD and a protonated nitrogen, which is present only on di- or mono-methylated H3K4. Therefore, LSD1 cannot demethylate tri-methylated lysines (H3K4me3). Kdm1 is found in co-repressor complexes like Co-REST, some of which silence neuronal genes in non-neuronal cells. By removing the activating methyl mark H3K4 and interacting with co-repressors, Kdm1 functions as a transcriptional repressor; nevertheless, interestingly it is associated with transcriptional activation as well. Kdm1 is important for androgen and estrogen receptor-mediated transcription in mammalian cells by functioning as an H3K9-specific demethylase (Shi and Whetstine, 2007). So far, Kdm1 is the only HDM known to be associated with both activation and repression of genes.
1.1.7.2 JmjC family histone demethylases

With the exception of Kdm1/LSD1, all other known HDMs to date belong to the JmjC family; they all contain the catalytic jumonji-C (JmjC) domain (Klose et al., 2006; Tsukada et al., 2010). JmjC domain proteins demethylate histones through a mechanism involving hydroxylation of the methyl groups and their subsequent release as formaldehyde (Tsukada et al., 2010). The JmjC domain is a variant of a common structural motif found from bacteria to mammals, the 2-histidine-1-carboxylate facial triad (Koehntop et al., 2005) that serves as a platform for binding divalent ions. Binding of an alpha-oxoacid cofactor (usually alpha-ketoglutarate) generates a highly reactive oxidizing intermediate that catalyzes substrate oxidation.

About 30 proteins with JmjC domains are annotated in the mouse and human genomes (Klose et al., 2006), although some of them are unlikely to be involved in histone demethylation due either to their cytoplasmic localization or to amino acid substitutions in the iron- and/or cofactor-binding sites (Cloos et al., 2008; De Santa et al., 2007; Klose and Zhang, 2007; Shi and Whetstine, 2007). These HDMs show high levels of specificity regarding both the target lysine they can modify and the level of methylation (mono-/di-/tri-methylation) they can reverse, implicating the diversity of functions they could be involved in (Table 2).
## Table 2: HDMs and their specificities

<table>
<thead>
<tr>
<th>HDM</th>
<th>Histone Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kdm5a/Jarid1a/RBP2</td>
<td>H3K4me2/3</td>
</tr>
<tr>
<td>Kdm5b/Jarid1b/PLU-1</td>
<td>H3K4me1/2/3</td>
</tr>
<tr>
<td>Kdm5c/Jarid1c/SMCX</td>
<td>H3K4me2/3</td>
</tr>
<tr>
<td>Jmjd1c/Trip8</td>
<td>H3K9</td>
</tr>
<tr>
<td>Kdm1/LSD-1/BHC110</td>
<td>H3K4me1/2, H3K9me1/2</td>
</tr>
<tr>
<td>Kdm3a/Jmjd1a/Jhdm2a</td>
<td>H3K9me1/2</td>
</tr>
<tr>
<td>Kdm3b/Jmjd1b/Jhdm2b</td>
<td>H3K9</td>
</tr>
<tr>
<td>Kdm4b/Jmjd2b</td>
<td>H3K9, H3K36me2/3</td>
</tr>
<tr>
<td>Jmjd2c/Gasc1</td>
<td>H3K9me2</td>
</tr>
<tr>
<td>Kdm4d/Jmjd2d</td>
<td>H3K9me2/3</td>
</tr>
<tr>
<td>Jarid2</td>
<td>H3K9me2/3</td>
</tr>
<tr>
<td>Kdm6b/Jmjd3</td>
<td>H3K27me2/3</td>
</tr>
<tr>
<td>Kdm6a/Utx</td>
<td>H3K27me2/3</td>
</tr>
<tr>
<td>Kdm2a/Jhdm1a/Fbx11</td>
<td>H3K36me1/2</td>
</tr>
<tr>
<td>Kdm2b/Jhdm1b/Fbxl-10</td>
<td>H3K36me1/2</td>
</tr>
<tr>
<td>Kdm4a/Jmjd2a/Jhdm3a</td>
<td>H3K9, H3K36me2/3</td>
</tr>
<tr>
<td>Jmjd6/Ptdsr</td>
<td>H3R2, H4R3</td>
</tr>
<tr>
<td>Jhdm1d</td>
<td>H3K9</td>
</tr>
<tr>
<td>Hif1an</td>
<td>(unknown)</td>
</tr>
<tr>
<td>Hr</td>
<td>(unknown)</td>
</tr>
<tr>
<td>Phf2/Jhdm1e</td>
<td>H3K9</td>
</tr>
<tr>
<td>Phf8/Jhdm1f</td>
<td>H4K20, H3K9</td>
</tr>
</tbody>
</table>
1.1.7.2.1 Kdm6b and H3K27-specific histone demethylases

Among the large family of JmjC domain-containing histone demethylases, only a small subfamily, consisting of Kdm6b/Jmjd3 and Kdm6a/Utx, shows substrate specificity for H3K27 methylation (Agger et al., 2007; De Santa et al., 2007). This subfamily contains one another member, Kdm6c/Uty, based on sequence similarity; however, Uty lacks enzymatic activity. Kdm6b shows high homology and structural relationship to Kdm6a/Uty, especially in the JmjC domain, but lacks the tetratricopeptide repeats (TPR) predicted to mediate protein-protein interactions (in fact, the N-terminal JmjC domain is the only clearly identifiable domain in Kdm6b/Jmjd3).

1.1.7.2.2 Kdm6b in development outside the nervous system

Kdm6b is highly expressed in differentiating macrophages, and is transiently associated with late HoxA gene promoters, an interaction that is believed to counteract PcG-mediated repression, to keep transcription active. On the other hand, in differentiated macrophages, Kdm6b levels are low and its occupancy on HoxA promoters is lost. An accompanying increase in H3K27me3 levels on the promoter and a drop in HoxA expression are seen (Agger et al., 2007; De Santa et al., 2007). Kdm6b regulation in macrophages is dynamic. Conversely, Kdm6a/Utx is expressed at constant low levels, suggesting that it behaves as a housekeeping gene (De Santa et al., 2007). In C. elegans the
Kdm6b ortholog XJ193 is required for proper gonadal development, although the specific mechanisms of regulation or target genes are not known (Agger et al., 2007). Kdm6a/Utx is also important for development. It regulates H3K27 methylation and the transcription of Hox genes in zebrafish, worms, and in human cell lines (Agger et al., 2007; Lan et al., 2007; Lee et al., 2007). Another instance where Kdm6b function is implicated is in the proper development of the epidermis – during keratinocyte differentiation, the loss of the H3K27me3 mark from differentiation gene promoters coincides with the binding of Kdm6b (Sen et al., 2008).

1.1.7.2.3 Kdm6b in neuronal development

Many neuronal genes are targeted by the PcG proteins and carry the repressive H3K27me3 mark in ES cells (Bernstein and Kellis, 2005; Boyer et al., 2006; Mohn et al., 2008). When ES cells differentiate into NSCs, the H3K27me3 mark is lost from a subset of neuronal genes (Mikkelsen et al., 2007), including neurogenins (Ngns), paired box gene 6 (Pax6), SRY-box containing 1 (Sox1), Sox3, NK2 transcription factor related locus 2 (Nkx2.2), achaete-scute complex homologue 1 (Ascl1 or Mash1), and Nestin. Kdm6b is shown to be required for expression of the neuronal genes Nestin, Pax6, and Sox1 (Burgold et al., 2008). Kdm6b is recruited to the Nestin promoter, where it removes the repressive H3K27me3 mark to activate Nestin expression. RNAi-mediated knockdown of Kdm6b in cultured ES cells results in the persistence of the H3K27me3 mark and
the suppression of Nestin expression. Similarly, the neurogenic genes Pax6 and Sox1 also require the binding of Kdm6b to their promoters for their expression in NSCs. Meanwhile, in embryonic NSCs, overexpression of Kdm6b is sufficient to induce the expression of a subset of neuronal genes (Dlx5, Gad1, Gad2, Dcx) (Jepsen et al., 2007). Kdm6b can bind to the Dlx5 promoter in a retinoic acid (RA)-dependent manner, and during RA-induced differentiation of NSCs the Dlx promoter undergoes demethylation of H3K27me3. During chick embryogenesis, BMP regulates the differentiation of multipotent neuroepithelial precursor cells to dorsal interneurons. In response to BMP signaling, Kdm6b interacts with Smad proteins and is recruited to the Noggin promoter, where it removes H3K27me3 to activate Noggin expression (Akizu et al., 2010; Dahle et al., 2010). For its requirement in neural lineage establishment, Kdm6b is an HDM of particular interest in the study of nervous system development.

1.1.7.3 Mechanisms of Kdm6b function

1.1.7.3.1 Inducible expression of Kdm6b

Kdm6b expression is induced by multiple factors in multiple cell types. In the nervous system, Kdm6b expression is induced in response to retinoid acid (RA) signaling (Jepsen et al., 2007). In NSCs, Kdm6b expression is repressed by the presence of the co-repressor SMRT on its promoter; during RA-dependent differentiation, SMRT is released from the Kdm6b promoter, allowing the ligand-bound RA receptor to activate Kdm6b transcription. In another example, in
striatal neurons, BDNF is sufficient to drive $Kdm6b$ expression via the MEK/ERK pathway (Gokce et al., 2009).

In macrophages, $Kdm6b$ shows rapid and transient induction in response to multiple stimuli. Toll-like receptor agonists (like LPS), INFγ, and the cytokine tumor necrosis factor alpha (TNFα) are all able to induce $Kdm6b$ expression in a transcription factor NF-KB-dependent manner (De Santa et al., 2009). Additionally, in macrophage activated by Th2 cytokines (eg: interleukin-4 and -13), $Kdm6b$ is induced via STAT-6-mediated signaling (Agger et al., 2009). Together, these data suggest that $Kdm6b$ expression is induced by various stimuli in multiple cell types, and that regulation of $Kdm6b$ expression might be a mechanism towards regulating its activity.

1.1.7.3.2 Recruitment of $Kdm6b$ to target loci

Histone modifiers like Kdm6b cannot directly bind DNA. Large complexes of cis-acting regulatory proteins or noncoding RNAs that engage DNA directly are probably responsible for the recruitment of these proteins to target gene sequences (Bernstein and Kellis, 2005). Both Kdm6b and Kdm6a have been identified in large regulatory protein complexes containing proteins that have H3K4-specific methyl transferase activity and proteins that can directly bind to DNA (Cho et al., 2007; De Santa et al., 2007; Issaeva et al., 2007). In lymphocytes, Kdm6b is recruited to target genes by T-box transcription factor T-bet (Miller et al., 2010). In a breast cancer cell line, Kdm6b associates with
estrogen receptor α (ERα) in a ligand-dependent manner to be targeted to enhancer regions (Svotelis et al., 2011). In the embryonic dorsal nerve cord, in response to BMP signaling, Kdm6b interacts with Smad proteins to be recruited to target genes (Akizu et al., 2010; Dahle et al., 2010). The destination of Kdm6b depends strongly on the presence of Kdm6b interactors that can recruit Kdm6b to specific target regions. Kdm6b seems to be targeted to both promoter and enhancer regions of genes. However, it is not known how Kdm6b is recruited to target promoters like Nestin promoter during ES cell differentiation or to target promoters during RA-dependent differentiation of NSCs. Also unknown are the proteins that interact with Kdm6b in differentiated neurons. As a demethylase that counteracts PcG repression, Kdm6b could be expected to be found in regulatory regions targeted by PcG, and in regulatory regions that show dynamic H3K27 methylation levels. Based on the only genome-wide ChIP (chromatin immunoprecipitation) analysis done for Kdm6b, in LPS-activated mouse macrophages, Kdm6b associates with the TSSs of active (bound by RNA Pol_II- and H3K4me3-positive) and inducible genes (De Santa et al., 2009). However, lack of Kdm6b in this system did not alter expression or H3K27me3 levels of many Kdm6b-bound genes. Therefore, the functional significance of Kdm6b in this scenario is not clear. More genome-wide studies in systems with established Kdm6b demethylases function are necessary to gain an accurate understanding of Kdm6b localization in the genome.
1.1.8 HDMs in disease

The perturbation of Kdm6b expression and activity, and hence potentially H3K27 methylation, has been implicated in carcinogenesis (Svotelis et al., 2011). Overexpression of Kdm6b has been shown to contribute to the pathogenesis of Hodgkin’s Lymphoma (Anderton et al., 2011). The INK4a/ARF tumor suppressor locus, a key executioner of cellular senescence, is silenced by PcG transcriptional repressors and Kdm6b acts as a tumor suppressor molecule by derepressing this specific locus, thus increasing the expression of INK4a and ARF in human and mouse fibroblasts, respectively (Agger et al., 2009; Barradas et al., 2009). Kdm6b expression is significantly decreased in several kinds of cancers (Agger et al., 2009; Cloos et al., 2008). Somatic mutations were also found in other H3K27-specific demethylase Kdm6a in multiple types of tumors (Dalgliesh et al., 2010; van Haaften et al., 2009). Together, these findings support a tumor suppressor role for the H3K27-specific demethylases.

Interestingly for the study of neurobiology, multiple HDMs have been associated with neurological disorders. One of the HDMs associated with multiple neurological disorders is the H3K4me3-specific demethylase Kdm5c/Jarid1c/Smcx. More than a dozen mutations of Kdm5c gene have been identified in individuals with intellectual disability (formerly known as mental retardation) (Tzschach et al., 2006). Many of these mutations reduce enzymatic activity. One such mutation is a non-sense mutation in the interaction domain
that results in the generation of a premature termination codon, which leads to reduced levels of Kdm5c transcript (probably due to nonsense-mediated mRNA decay) and therefore to reduced activity (Santos-Rebouças et al., 2011). Genes regulated by Kdm5c, like sodium channel type 2A (Scn2a) and synapsin (Syn1), are themselves implicated in epilepsy, autism, and schizophrenia (Tahiliani et al., 2007). It is possible that mutations in Kdm5c lead to alterations in Kdm5c function, causing disregulation of target genes whose gene products are directly involved in regulating neuronal activity. Also, studies in zebrafish and cultured primary neurons suggest roles for Kdm5c in neuronal cell survival and dendritic development (Iwase et al., 2007). Abnormalities of neuronal morphology, including dendritic development, have been reported in patients with intellectual disability (Fiala et al., 2002; Ropers and Hamel, 2005). Another H3K9-specific HDM associated with intellectual disability is Phf8/Jhdm1f (Laumonnier et al., 2005). Truncating mutations that occur in or near the JmjC domain have been identified in patients, showing the importance of demethylase function for proper neuronal differentiation and/or function. Kdm6b and Kdm5a (genes that encode for H3K27- and H3K4-specific demethylases, respectively) have been identified as candidate genes for intellectual disability as presumably causative mutations were found in these genes (Najmabadi et al., 2011). Together, these studies suggest that HDMs play important functions in neuronal development and function and that disruption of their function leads to disease.
1.2 The cerebellum

1.2.1 The cerebellum as a model system for neuronal molecular genetics

The generation and maturation of CGNs begins during embryonic life but continues for several weeks after birth (Wang and Zoghbi, 2001). This prolonged post-natal development and several other attributes of cerebellar development make CGNs an ideal cell type for studying the molecular mechanisms that guide neuronal differentiation. In the cerebellar cortex, CGNs account more than 99% of neurons and 85% of all cells (Altman and Bayer, 1997). Committed CGN precursors can be purified in large quantities to >90% homogeneity from postnatal cerebellum (Hatten and Heintz, 1995), and cultured under conditions that recapitulate their differentiation through defined stages (dividing precursor, immature neuron, mature neuron) facilitating experimental investigation of the molecular mechanisms of this process.

1.2.2 Cerebellar circuitry

Sensory inputs from the periphery, about the position and movements of the body, are first sent to the pre-cerebellar system, a group of nuclei in the brain stem. Most of these nuclei (except for the inferior olivary nucleus that projects climbing fibers directly to the Purkinje cells) project to the CGNs that in turn project CGNs to Purkinje cells. The Purkinje cells are the primary output neurons from the cerebellar cortex, and they project to the deep cerebellar nuclei. These
nuclei project to the cerebral cortex to mediate the fine control of motor movement and balance (Voogd and Glickstein, 1998).

CGNs are glutamatergic (uses glutamate as their transmitter), excitatory neurons. The other prominent type of neuron in the cerebellum, Purkinje neurons are GABAergic [uses GABA (γ-aminobutyric acid) as their transmitter] neurons. The other classes of neurons present in the cerebellum are golgi cells, stellate cells, and basket cells. All three classes of neurons release GABA, golgi cells additionally release glycine. Golgi cells modulate CGN function and the stellate and basket cells modulate Purkinje neuron output.

1.2.3 Diseases of the cerebellum

The cerebellum is commonly associated with its role in motor control, and many diseases of the cerebellum produce ataxia (Schmahmann, 2004). In addition, the cerebellum has been implicated in higher cognitive functions as well (Middleton and Strick, 1998). The prolonged development of the cerebellum beginning at embryonic stages but continuing into several weeks following birth, renders it vulnerable to environmental insults that may contribute to neuropsychiatric impairments as well. For example, anatomical and/or functional defects of the cerebellum have been associated with neuropsychiatric disorders of childhood including attention-deficit hyperactivity disorder, autism, and schizophrenia (Andreasen and Pierson, 2008).
1.2.4 Cerebellar development

Neuronal cells develop through a sequence of temporal events consisting of proliferation, differentiation, migration, and maturation. The most abundant neurons in the cerebellum, the cerebellar granule neurons (CGNs), are derived from the committed precursors which migrate from the rhombic lip in the hind brain to form a secondary proliferative zone in the external granule cell layer (external EGL) at the outer layer of the cerebellum (Altman and Bayer, 1997). During the early postnatal period, granule cell progenitors (GCPs) proliferate extensively in the outer EGL. At P3 GCPs leave the cell cycle and begin to differentiate in the inner-part of the EGL (inner EGL). From P7-P15 CGNs migrate inwards across the molecular layer to form the internal granule cell layer (IGL), where they reach terminal maturity (Wang and Zoghbi, 2001). Due to this developmental migration, the EGL is the thickest at P8 (7-10 layers), decreases to 1-2 layers at P14, and disappears by P21. By P21 the CGNs of the IGL have made synaptic connections and matured to a level that they are indistinguishable from the adult CGNs.

Precise regulation of this process requires the actions of brain-derived neurotrophic factor (BDNF) (Huang and Reichardt, 2001; Lindholm and Thoenen, 1993; Schwartz et al., 1997). BDNF influences CGN development by promoting CGN progenitors exit from the cell cycle, promoting survival, acting as a chemokinetic factor to induce CGN migration, functioning as a chemotactic factor...
to ensure that the migrating CGNs are moving in the correct orientation, and enhance axonal outgrowth and drive gene expression programs of CGN maturation (Bonni et al., 1999; Borghesani et al., 2002; Choi et al., 2005; Lindholm and Thoenen, 1993; Segal et al., 1995; Suzuki et al., 2005; Zhou et al., 2007).

Figure 1: Postnatal cerebellar development
1.3 *Research objectives*

The goal of this study was to advance the understanding of how chromatin regulation contributes to aspects of neuronal biology. Starting from a simple screen, I have made a unique contribution to the understanding of histone demethylases in neurons by identifying the expression, regulation, and functions of Kdm6b.

Histone modifications associated with the regulatory regions of a gene are important determinants of the transcriptional activity of that gene. Histone methylation can occur at multiple residues on multiple histones and has been associated with both transcriptional activation and repression. These data point to histone methylation as a particularly information-rich modification. However, for a long time, histone methylation was thought of as a permanent mark not amenable to regulated alterations, which limited interest in the study of this modification. Not long before I started my thesis a large family of histone demethylases were identified. The functions of some of these enzymes, especially in early development and in cancer, are beginning to be revealed through molecular genetic studies. However, at the time I started this work essentially nothing was known about the function of these enzymes in the nervous system.

Once the stability of the methyl mark is combined with the ability to selectively remove them, histone methylation becomes a potential mechanism of
cell plasticity. During development, the stability of the mark makes it suitable for the establishment of lineage-specific-gene expression programs. However, the ability to remove these marks is important in later stages of development for the changes in cell-type specific gene expression that accompany cell differentiation. Additionally, in mature neurons, the ability to dynamically regulate histone methylation could allow for stimulus-dependent function and plasticity of regulated gene expression by this mechanism.

To determine which histone demethylases might be of particular interest to CNS plasticity, we first started with the assumption that regulation of expression of an enzyme itself is a way to regulate its function. Thus I began my thesis with a screen for activity-induced histone demethylases in vivo. These data are described in chapter 2 in which I show that four histone demethylases out of the large histone demethylase family show upregulation of their expression in the hippocampus following seizure, a well-defined model of neuronal activity. As a result of this screen we focused on the H3K27 histone demethylase Kdm6b, which shows robust activity-dependent induction and high levels of expression in the developing brain. Using RNA interference to study functional roles of Kdm6b in cerebellar granule neurons as a model for neuronal differentiation, in chapter 3 I show that I have now identified functions for Kdm6b in cerebellar granule neuron survival and differentiation.
2. Activity-induced expression of *Kdm6b*

2.1 Summary

Changes in gene transcription driven by the activation of intracellular calcium signaling pathways play an important role in neural development and plasticity. Stimulus-driven alteration of histone modifications is one way of modulating neuronal activity-regulated gene transcription. Histone modifying enzymes may themselves be regulated by neuronal activity as a means to altering histone modifications. We induced seizures in mice and screened for histone demethylases (HDMs) – enzymes that can remove methyl groups from histones – whose expression is induced in the hippocampus. We focused on the H3K27-specific HDM *Kdm6b*, which showed the highest induction and has previously been implicated in neuronal lineage establishment. *In situ* hybridization analysis revealed that *Kdm6b* is highly induced in post-mitotic neurons of the dentate gyrus region of the hippocampus. We can recapitulate the activity-dependent induction of *Kdm6b* expression in cultured hippocampal neurons by application of Bicuculline, a GABA<sub>A</sub> receptor antagonist that leads to synaptic NMDA receptor activation and calcium influx. *Kdm6b* expression is also induced following application of BDNF, a neurotrophic factor that is upregulated in the hippocampus following seizure.
2.2 Introduction

The structure and function of neurons in the brain can change in response to alterations in neuronal activity. One important mechanism through which changes in activity can induce various forms of neural plasticity is through the regulation of gene expression. It is well known that the post-translational modifications of the histone proteins associated with a gene can influence the transcriptional state of that gene. Furthermore, numerous studies have identified activity-dependent modifications to histone proteins at genes whose expression is known to be regulated by neuronal activity. For example, electroconvulsive treatment induces the expression of several neuronal activity-regulated genes including c-fos, and concurrent alterations in histone phosphorylation are observed at the c-fos promoter (Borrelli et al., 2008; Tsankova et al., 2004). Additionally, acute cocaine treatment induces Ser10/Lys14 H3 phospho-acetylation, a modification associated with gene activation, at the c-fos promoter, and it has been shown that c-fos expression is induced in response to this stimulus (Borrelli et al., 2008). On the other hand, chronic cocaine decreases the expression of c-fos and is associated with an increase in the repressive H3K9 methyl mark at the c-fos gene (Borrelli et al., 2008; Kumar et al., 2005; Renthal and Nestler, 2008). Finally, genes induced by chronic cocaine, Cdk5 and Bdnf, are associated with increased H3 acetylation, an active modification, following this treatment (Borrelli et al., 2008; Kumar et al., 2005). Taken together, these studies demonstrate that post-translational modifications of the histones
associated with a particular gene might serve as one important mechanism of altering the expression level of that gene. In the nervous system, one of the best-studied activity-dependent histone modifications is acetylation. Alterations in histone acetylation are mediated by histone acetyltransferases (HATs) that add acetyl groups to histones and histone deacetylases (HDACs) that remove acetyl groups from histones. Several different molecular mechanisms have been identified that can modulate histone acetylation in response to changes in neuronal activity. For example, once activated by neuronal activity-induced phosphorylation, the transcription factor cAMP/calcium response element binding protein (CREB) recruits CREB-binding protein (CBP), a co-activator with intrinsic HAT activity, to target gene promoters (Lonze and Ginty, 2002). CBP recruitment in turn induces histone acetylation and transcriptional activation of CREB target genes. Additionally, CBP itself is phosphorylated and activated by several kinases in response to neural activity (Riccio, 2010). Increases in intracellular calcium levels following neural activity activates the Ca^{2+}/calmodulin-dependent protein kinases, which can phosphorylate class II HDACs. This phosphorylation provides a docking site for the protein 14-3-3, which mediates the export of the phosphorylated HDACs from the nucleus, thus decreasing HDAC activity and increasing gene transcription (Borrelli et al., 2008; Renthal and Nestler, 2008). Neural activity can also modulate histone acetylation by regulating the intracellular distribution of HDACs. In hippocampal neurons, nuclear export of HDACs has been shown to occur following the activation of
NMDA receptors (Riccio, 2010), thereby decreasing HDAC activity in the nucleus and repression of target genes. Finally, following chronic cocaine treatment, HDAC1 is recruited to c-fos, leading to the deacetylation of histones at that site and a decrease in c-fos expression. This stimulus also induces HDAC5 phosphorylation and nuclear export (Dulac, 2010). Thus, the complex activity-dependent regulation of numerous HATs and HDACs can specifically alter the expression patterns of distinct sets of target genes via regulation of histone acetylation at those genes.

In contrast to mechanisms of regulation of histone acetylation, comparatively little is known about how histone methylation can change in response to neural activity. Histone demethylases (HDMs) are enzymes that can remove methyl marks from histones. A large family of HDMs containing the catalytic JmjC domain has been relatively recently identified (Cloos et al., 2008). Given the high thermodynamic stability of histone methyl marks, this particular histone modification was originally thought of as a permanent mark that would persist through a cell’s lifetime. The discovery of demethylases raised the exciting possibility that methyl marks are dynamically regulated and possibly in response to stimuli like neuronal activity. While the role of HDMs in activity-dependent gene transcription remains largely unknown, many of these proteins have been linked to mental disease, suggesting that they have important functions in the nervous system.
To determine which HDMs might be regulated by neuronal activity, we assessed their expression in the hippocampus following seizure, a model for robust neural activity. The H3K27-specific HDM *Kdm6b*, which is known to function in neuronal lineage establishment (Burgold et al., 2008; Jepsen et al., 2007), showed the most robust upregulation in expression following seizure. By *in situ* hybridization analysis we determined that *Kdm6b* is upregulated in the post-mitotic neurons of the DG region of the hippocampus. *In vitro, Kdm6b* expression can be induced by stimuli that are known to drive synaptic NMDA receptor activation and by the neurotrophin BDNF. These *in vivo* and *in vitro* studies raise the intriguing possibility that Kdm6b could contribute to activity-regulated changes in gene transcription that are important for various forms of neuronal plasticity.

**2.3 Results**

**2.3.1 *Kdm6b* expression in the hippocampus shows robust upregulation following seizure**

In order to identify HDMs whose expression is regulated in response to neuronal activity, and therefore might regulate activity-dependent gene transcription in the nervous system, we examined the expression levels of several HDMs in the hippocampus following pilocarpine-induced seizure. We isolated hippocampal mRNA from the brains of control mice and mice that had undergone pilocarpine-induced seizure, and analyzed gene expression by
Figure 2: *Kdm6b* expression shows robust upregulation following seizure. Adult C57/B6 mice were injected with saline (control), or pilocarpine (375mg/kg) to induce seizure. Mouse hippocampi were dissected under control conditions (none) or 1, 3, or 6hrs following pilocarpine-induced status epilepticus. RNA was harvested, cDNA synthesized, and SYBR green PCR used to quantify gene expression. All data were normalized to expression of the housekeeping gene *Gapdh* as control for sample handling. Data are expressed as fold induction over control. *p < 0.05, error bars represent S.E.M., n=5-6
quantitative real-time PCR. We used the *Bdnf* gene as a control for the ability of this seizure paradigm to induce programs of gene transcription. Consistent with what has been reported in literature (Binder and Scharfman, 2004; Ploski et al., 2006), we see robust upregulation of *Bdnf* within the first three hours following seizure (data not shown). Of all the HDMs tested, H3K27-specific HDM *Kdm6b* showed the most robust increase in expression (Figure 2). In contrast, the close family member and H3K27me3-specific HDM *Kdm6a* showed no changes in expression. Other HDMs that also showed significant levels of induction are *Jmjd1c/TRIP8, Jarid2*, and *Kdm7a/Jhdm1d*. These data demonstrate that *Kdm6b* is a HDM whose expression can be highly induced by increased neuronal activity; therefore, we decided to further explore its role in the nervous system.

### 2.3.2 Seizure induces *Kdm6b* expression in post-mitotic neurons of the DG of the hippocampus

Our data obtained from analyzing the brains of mice that had undergone seizure suggests that increases in neuronal activity leads to the upregulation of *Kdm6b* expression. Since we used RNA extracted from the whole hippocampus, we were unable to determine the regional distribution of this upregulation within the hippocampus. To identify the hippocampal substructures where *Kdm6b* mRNA levels are altered following seizure, we used *in situ* hybridization to assess the spatial distribution of *Kdm6b* mRNA induction. *In situ* hybridization was performed using radioactive riboprobes targeting the sense or antisense strand of *Kdm6b* exon III. *Bdnf* was used as a control because the pattern and
Figure 3: Upregulation of *Bdnf* and *Kdm6b* in the DG following seizure. Adult C57/B6 mice were injected with saline (control), or pilocarpine (375mg/kg) to induce seizure. Mouse hippocampi were extracted 3hrs after injection with saline (control) or 3hrs following pilocarpine-induced status epilepticus (seizure). *In situ* hybridization was carried out using radiolabeled antisense riboprobes targeting either *Bdnf* or *Kdm6b*. Slides were subsequently processed for emulsion autoradiography. Cresyl violet-staining shows the location of the cells. Molecular layer on top.
extent of *Bdnf* upregulation by seizure are well described (Binder and Scharfman, 2004; Ploski et al., 2006). As before, we subjected adult mice to pilocarpine-induced seizure. Brains were extracted from mice sacrificed 3 hours following the onset of status epilepticus (SE). *Kdm6b* mRNA showed upregulation in the dentate gyrus (DG) region of the hippocampus (Figure 3). Sense controls yielded only non-specific background labeling, indicating that our *in situ* hybridization is specific for the *Kdm6b* mRNA sequence. As was previously reported, we also see the upregulation of *Bdnf* in the DG (Figure 3), as well as in the CA1 and CA3 regions of the hippocampus (Binder and Scharfman, 2004). The *Kdm6b in situ* signal was seen throughout the layers of the DG of the hippocampus, suggesting that *Kdm6b* may be important for the cellular adaptations that occur in the DG following seizure.

The mouse DG is a known site of adult neurogenesis (Alvarez-Buylla and Lim, 2004). The neural precursor cells (NPCs), which are known to proliferate and make new neurons following SE, reside in the inner face of the DG in the subventricular zone (SVZ) (Parent and Lowenstein, 2002). Expression of *Kdm6b* in these NPCs would raise the possibility that *Kdm6b* is involved in seizure-induced adult neurogenesis. To determine whether *Kdm6b* is expressed in the NPCs, we looked for colocalization of *Kdm6b* and the proliferation marker Ki67. Since we do not have a reliable Kdm6b antibody for immunostaining, we combined fluorescent *in situ* hybridization for *Kdm6b* with immunostaining for Ki67. If *Kdm6b* is expressed in NPCs following seizure, we would predict that the
Figure 4: Upregulation of Kdm6b following seizure occurs outside the proliferating progenitors in the DG. Adult C57/B6 mice were injected with saline (control), or pilocarpine (375mg/kg) to induce seizure. Mouse hippocampi were extracted 3hrs after injection with saline (control) or 3hrs following pilocarpine-induced status epilepticus (seizure). Fluorescent in situ hybridization was carried out using antisense riboprobes targeting Kdm6b. Immunostaining was done using antibodies specific to Ki67 to mark progenitors. Kdm6b shown in red, Ki67 in green, and DAPI in blue. Molecular layer on top.
proliferation marker Ki67 would colocalize with Kdm6b. However, our analysis showed no colocalization of the signals, indicating that Kdm6b expression is not upregulated in the NPCs following seizure (Figure 4). This lack of Kdm6b expression in the NPCs suggests that Kdm6b is not involved in seizure-induced neurogenesis. However, robust upregulation of Kdm6b was seen in the rest of the DG, in post-mitotic neurons that do not stain for Ki67. These data demonstrate that Kdm6b is not involved in adult neurogenesis following seizure, and raise the possibility that Kdm6b might be involved in activity-dependent forms of hippocampal plasticity in post-mitotic neurons.

2.3.3 Stimuli known to activate synaptic glutamate receptors is sufficient to drive Kdm6b expression

Our data showing increased Kdm6b expression levels following seizure suggest that Kdm6b expression is induced by neuronal activity. To identify signaling mechanisms that might govern neuronal activity-induced Kdm6b expression, we used pharmacological manipulations to mimic neuronal activity in dissociated post-mitotic hippocampal neurons in culture. We assessed changes in Kdm6b transcription following these treatments with quantitative real-time PCR.

We first mimicked increased synaptic firing using two methods: bicuculline and 4-AP or tetrodotoxin (TTX) withdrawal. Bicuculline is a GABA\textsubscript{A} receptor antagonist that, when applied along with the potassium channel blocker 4-AP, leads to synaptic NMDA receptor activation and calcium influx (Hardingham et
al., 2002). Treating cells with the sodium channel blocker TTX for 48 hours and washing TTX out of the media is another way to increase synaptic NMDA receptor activity. A gene known to be induced by both of these two modes of neuronal activity is *Bdnf*. Induction of *Bdnf* following TTX withdrawal is reduced by the NMDA receptor blocker APV or treatment with L-VSCC blocker nimodipine. When used in combination, these blockers completely abolish *Bdnf* induction by TTX withdrawal, indicating that transcriptional activity following TTX withdrawal is due to the activation of both NMDA receptors and L-VSCCs (Lyons, 2001). Both modes of enhancing synaptic activity lead to the significant upregulation of *Kdm6b* in cultured hippocampal neurons (Figure 5). We found that this upregulation is transient, with peak *Kdm6b* expression occurring after 3 hours of treatment and expression levels returning to baseline by 6 hours. These data suggest that activation of synaptic NMDA receptors is sufficient to drive the expression of *Kdm6b*. To determine whether activation of L-VSCCs might contribute to this upregulation, we next used high KCl-mediated membrane depolarization.

Neuronal activity leads to membrane depolarization and the opening of L-VSCCs (West and Greenberg, 2011). Membrane depolarization can be achieved in culture through the addition of a high concentration of KCl. To test whether activation of L-VSCCs can drive *Kdm6b* expression, we added KCl to culture media to achieve a final concentration of 55mM KCl and incubated the culture for 3 or 6 hours before harvesting for RNA. This procedure was sufficient to drive
Figure 5: Activation of glutamatergic synaptic transmission is sufficient to increase \textit{Kdm6b}. A. Addition of the GABA\textsubscript{A} receptor antagonist bicuculline (1\textmu M) along with the K channel blocker 4AP to cultured hippocampal neurons (E18 + 14DIV) is sufficient to increase \textit{Kdm6b} mRNA levels. B. TTX withdrawal, which activates synaptic glutamate receptors, is also sufficient to induce \textit{Kdm6b} expression. C. Membrane depolarization, which opens L-type VSCCs, is not sufficient to induce \textit{Kdm6b} expression. Following each treatment RNA was isolated, cDNA synthesized, and real time PCR run with primers specific to \textit{Kdm6b}. All data were normalized to expression of the housekeeping gene \textit{Gapdh} as control for sample handling. Data are expressed as fold induction over (A) none, (B) TTX 48 hrs, and (C) unstim. *p < 0.05, error bars represent S.E.M., n=3-12 per group.
the expression of Bdnf and Fos (data not shown). However, it failed to induce Kdm6b expression at any of the time points tested, demonstrating that signaling cascades activated by calcium influx through L-VSCCs are not sufficient to drive Kdm6b expression (Figure 5). The induction of Kdm6b we see following TTX withdrawal is probably due to the activation of NMDA receptors and not the activation of L-VGCCs, although this remains to be tested using inhibitors that specifically block either pathway. Together these data suggest that the particular route of calcium entry can determine neuronal activity-dependent induction of Kdm6b expression.

2.3.4 BDNF induces transcription of Kdm6b in cultured hippocampal neurons

The level of induction that is observed with stimuli that activate synaptic NMDA receptors in culture is noticeably less than the level of induction that is seen in vivo, suggesting that other signaling cascades may contribute to Kdm6b induction in vivo following seizure. The expression of the neurotrophic factor BDNF is known to be induced by neuronal activity, and in particular, is robustly induced by seizure. Additionally, through interaction with its high affinity receptor TrkB, BDNF can also regulate gene expression programs (Kaplan and Miller, 2007). Thus, BDNF-TrkB signaling appeared to be a good candidate pathway that could facilitate Kdm6b expression following seizure.

To determine whether BDNF can drive Kdm6b expression, we incubated hippocampal cultures in the presence or absence of BDNF for 3 hours. The
presence of BDNF significantly increased $Kdm6b$ mRNA as compared to an untreated control (Figure 6). To determine whether this increase in mRNA is due to increased transcription, we cultured cells in the presence of the transcription inhibitor actinomycin D (ActD). The presence of ActD alone decreased $Kdm6b$ mRNA levels below control conditions, suggesting that $Kdm6b$ mRNA is low in stability and has a high turnover rate. Addition of ActD to BDNF cultures inhibited BDNF-induced upregulation (Figure 6). Taken together, these data suggest that BDNF drives $Kdm6b$ expression in a transcription-dependent manner in cultured hippocampal neurons. Based on this, we hypothesize that BDNF signaling may be partially contributing to the induction of $Kdm6b$ seen in the hippocampus following seizure.

2.4 Discussion

2.4.1 Seizure-induced expression of HDMs

In this study we used pilocarpine-induced seizure as a paradigm to identify HDMs whose expression is regulated by neuronal activity. We've identified four demethylases as neuronal activity regulated genes – $Kdm6b/Jmjd3$, $Jmjdc$, $Jhdmc2$, $Jarid2$, and $Kdm7a/Jhdmd$. Interestingly, three of these genes encode demethylases that specifically regulate H3K9 methylation. Their inducibility following neuronal activity suggests that modulation of H3K9 methylation is an important mode of regulating gene expression in response to neuronal activity. These four genes encode for HDMs with known specific demethylase activity and have been functionally implicated in multiple cellular
Figure 6: BDNF is sufficient to increase Kdm6b transcription. Cultured embryonic rat hippocampal neurons at DIV 7 were left untreated or treated with either BDNF (50ng/ml), transcriptional inhibitor actiomycin D (ActD), or BDNF + ActD for 3hrs. RNA was harvested, cDNA synthesized, and real time PCR run with primers specific to Kdm6b. Data are expressed as fold induction over none. *p < 0.05, error bars represent S.E.M., n=3 per condition.
systems. *Jmjd1c* was originally described for its interaction with the thyroid hormone receptor (TR) (Cloos et al., 2008). A variant of *Jmjd1c* can interact with and is a co-activator of androgen receptor (AR) (Wolf et al., 2007) and in the brain, is present in AR-expressing neuronal populations. Importantly, a chromosomal aberration that leads to decreased expression has implicated *Jmjd1c* as a candidate gene for autism (Castermans et al., 2004). A possible tumor suppressor role has also been ascribed to *Jmj1c*, due to the finding that there is reduced *Jmjd1c* expression in breast cancer tissue (Wolf et al., 2007). While comparatively little is known about *Kdm7a*, it has been assigned a tumor suppressor role since its expression can suppress tumor growth by down-regulating angiogenesis (Osawa et al., 2011). Additionally, evidence that inhibition of *Kdm7a* zebrafish orthologs leads to developmental brain defects suggests that *Kdm7a* may have roles in neural differentiation (Tsukada et al., 2010). *Jarid2*, another JmjC family member, was the first protein in which the jumonji (Jmj) domain was identified. The term ‘jumonji’ actually means cruciform in Japanese and the gene was so named because mice with gene trap insertion in this locus develop an abnormal cross-like structure in the neural tube. *Jarid2* is also referred to as *Jumonji* (Takeuchi et al., 2006). *Jarid2* null mice die in utero or neonatally due to heart abnormalities (Cloos et al., 2008). Importantly, *Jarid2* is identified as a candidate gene for schizophrenia (Pedrosa et al., 2007). Biological functions of the H3K27-specific demethylase *Kdm6b* will be discussed in detail later since we focus on this protein in our study.
While each of these genes are involved in many cellular events in many cell types including neurons, ours is the first data to show that they are inducible following neuronal activity. Regulated HDM expression suggests that the histone methyl marks they regulate, H3K9 and K3K27, could be dynamically regulated in response to neuronal activity in the adult hippocampus. In turn, these modifications determine how target genes are expressed and allow for long term neuronal plasticity. Because both of these marks are repressive marks, increasing the expression of these demethylases should lead to enhanced activation of gene transcription. While targets of these HDMs in the adult hippocampus remain to be identified, regulation of the expression of a particular HDM is only one way of modulating the enzymatic activity of that HDM. Post-transcriptional modifications of the RNA transcript, post-translational modifications of the protein, regulation of nuclear-cytoplasmic shuttling, regulation through binding partners, and regulation of the recruitment to target genes are other ways through which HDM enzymatic activity can be modulated. For example, Kdm1 is alternatively spliced to generate a neurospecific isoform (Zibetti et al., 2010). Kmd1 function is also regulated at the protein level, and can function either as an activator or a repressor depending on its binding partners (Cloos et al., 2008). Additional mechanisms of regulating the enzymatic activity of the four demethylases that we have discussed here, other than at the level of expression, remain to be identified.
Out of the demethylases that showed significantly increased expression following seizure, \textit{Kdm6b} showed the most robust upregulation. \textit{Kdm6b} is a histone H3K27-specific demethylase important for neural lineage establishment (Burgold et al., 2008; Jepsen et al., 2007). \textit{Kdm6b} is shown to be required for the expression the neuronal genes \textit{Nestin}, \textit{Pax6}, and \textit{Sox1} that are required for the establishment of a neural fate. In ES cells differentiating to a neural lineage, \textit{Kdm6b} is recruited to the \textit{Nestin} promoter, where it removes the repressive H3K27me3 mark to activate \textit{Nestin} expression. RNAi-mediated knockdown of \textit{Kdm6b} in cultured ES cells results in the persistence of H3K27me3 mark and the suppression of \textit{Nestin} expression (Burgold et al., 2008). Similarly, the neurogenic genes \textit{Pax6} and \textit{Sox1} also require the binding of \textit{Kdm6b} to their promoters for their expression in NSCs. Meanwhile, in embryonic NSCs, overexpression of \textit{Kdm6b} is sufficient to induce the expression of a subset of neuronal genes (\textit{Dlx5}, \textit{Gad1}, \textit{Gad2}, \textit{Dcx}) (Jepsen et al., 2007). \textit{Kdm6b} can bind to the \textit{Dlx5} promoter in a retinoic acid (RA)-dependent manner and during RA-induced differentiation of NSCs the \textit{Dlx5} promoter undergoes demethylation of H3K27me3. During chick embryogenesis, \textit{Kdm6b} is important for the induction of a dorsal interneuron fate (Akizu et al., 2010). For its requirement in neural lineage establishment \textit{Kdm6b} is a HDM of particular interest in the study of nervous system development.

\textit{Kdm6b} function has also been shown to be important in developmental processes in non-neural systems. \textit{Kdm6b} is highly expressed in differentiating
macrophages, and is transiently associated with late HoxA gene promoters, an interaction that is believed to counteract PcG-mediated repression, to keep transcription active. On the other hand, in differentiated macrophages, Kdm6b levels are low and its occupancy on HoxA promoters is lost. An accompanying increase in H3K27me3 levels on the promoter and a drop in HoxA expression are seen (Agger et al., 2007; De Santa et al., 2007). In zebrafish the Kdm6b ortholog XJ193 is required for proper gonadal development, although the specific mechanisms of regulation or target genes are not known (Agger et al., 2007). Another instance where Kdm6b function is implicated is in the proper development of the epidermis – during keratinocyte differentiation, the loss of the H3K27me3 mark from differentiation gene promoters coincides with the binding of Kdm6b (Sen et al., 2008).

Kdm6b is shown to be a dynamically expressed gene in multiple cell types. As mentioned above, Kdm6b expression changes over the course of macrophage differentiation. Also, Kdm6b is quickly and strongly induced in macrophages in response to bacterial products or inflammatory cytokines such as lipopolysaccharides (LPS). In the case of LPS, interaction with Toll-like receptors leads to activation of Kdm6b expression through mechanisms dependent on the transcription factor NF-KB. The cytokine tumor necrosis factor alpha (TNFα) is also able to induce Kdm6b expression in macrophages, but to a lesser extent (De Santa et al., 2007). In THP-1 cells TNFα can induce Kdm6b (Li et al., 2008). Kdm6b promoter contains several binding sites for NF-KB. Th2
cytokines (eg: interleukin-4 and -13) induce Kdm6b in macrophages by STAT-6-mediated signaling (Agger et al., 2009). Different cell types employ different signaling pathways to induce Kdm6b expression. Signaling through NF-KB could be a signaling event relevant in neurons in terms of Kdm6b induction. In neurons, NF-KB is activated by membrane depolarization and glutamate stimulation (West and Greenberg, 2011), suggesting a conserved role for NF-KB-mediated signaling in the induction of Kdm6b expression in different cell types.

Importantly, the only other known H3K27-specific HDM and close family member of Kdm6b, Kdm6a, was not upregulated by seizure. Studies in non-neural systems have also suggested that while Kdm6b is a dynamically regulated gene, Kdm6a levels stay constant even under the same stimuli that induce Kdm6b expression, which has lead to the suggestion that Kdm6a has more of a “housekeeping” role. These observations suggest that these two demethylases are regulated by distinct mechanisms, and may have non-overlapping functions in the nervous system.

2.4.2 Neuronal activity-induced Kdm6b expression occurs in the DG region of the hippocampus

The DG region of the hippocampus showed robust Kdm6b expression compared to the other regions of the hippocampus. The DG has been identified as a site of adult neurogenesis (Alvarez-Buylla and Lim, 2004), and seizure is a stimulus that can potentiate this process (Parent and Lowenstein, 2002). Two different studies examining the role of Kdm6b in the nervous system have
implicated it in neurogenesis. *Kdm6b* is necessary for the establishment of NSCs, and important for the expression of neuronal genes in NSCs differentiating into neurons (Burgold et al., 2008; Jepsen et al., 2007). Together these observations led us to hypothesize that Kdm6b may be involved in neurogenesis following seizure. We further hypothesized that Kdm6b would be upregulated in the neural progenitor cells (NPCs) of the DG following seizure. However, to our surprise, *Kdm6b* is not upregulated in the NPCs of the DG, suggesting that Kdm6b is not involved in the proliferation of NPCs in the DG following seizure when there is rapid proliferation of NPCs. The exclusion of *Kdm6b*, which promotes neuronal differentiation, from NCPs could be necessary for preserving the proliferation potential of NCPs.

### 2.4.3 Neuronal activity-induced *Kdm6b* expression *in vitro*

We find that L-VSCC activation is not sufficient but NMDA receptor activation is sufficient for *Kdm6b* induction in cultured hippocampal neurons. This result is not particularly surprising because calcium influx through different types of channels has different abilities to activate gene transcription, and there are indeed differences in calcium influx through L-VSCCs vs. through NMDA receptors. The difference in ability of L-VSCCs and NMDA receptors to induce *Kdm6b* expression may lie in the distinct manners in which intracellular calcium increases following their activation and in the distinct signaling molecules that are activated. For example, calcium influx through L-VSCCs is tightly coupled to the activation of CREB (Bading et al., 1993), whereas calcium influx following NMDA
receptor activation is coupled to SFR mediated gene expression (Bading et al., 1993; Hardingham et al., 1997). Also, local calcium increases following the activation of L-VSCCs could lead to the activation of CaM, a calcium sensor that can directly interact with L-VGCCs, and subsequent translocation to the nucleus to regulate gene expression (Deisseroth et al., 1998; Dolmetsch et al., 2001). On the other hand, the large intracellular C-termini of NMDA receptor subunits allow for protein-protein interactions with signaling molecules. Pharmaceutical manipulation to block the functions of specific downstream signaling molecules will be necessary to identify the signaling mechanisms involved in the activation of Kdm6b expression.

2.5 Experimental methods

2.5.1 Pilocarpine-induced seizure

Adult (8-12 week old) wildtype C57BL6/J mice were weighed and injected with 1mg/kg methyl scopolamine nitrate intraperitoneally (i.p.). 30 minutes later mice were injected with either 337mg/kg pilocarpine HCl or saline (control mice) i.p. (both scopolamine and pilocarpine were diluted in 0.9% injectable NaCl). Stock solutions were prepared such that each animal received 0.1cc of drug. Scopolamine was administered to reduce the peripheral side effects of pilocarpine and the risk of death upon pilocarpine injection. Following pilocarpine injection, animals were monitored for status epilepticus (defined as a continuous limbic motor seizure of stage 2 or higher). Status epilepticus was allowed to proceed for 3 hrs and then was terminated by administration of diazepam.
Pilocarpine-treated animals that failed to develop or did not survive status epilepticus were excluded from the study. At various times after pilocarpine-induced status epilepticus (1–6 hrs after the initiation of the seizure by pilocarpine injection), animals were anesthetized with isofluorane and sacrificed by decapitation prior to tissue harvesting. Control mice were never given diazepam and were sacrificed 3 hrs after receiving scopalamine. Following decapitation, hippocampi were dissected out as previously described (McDowell et al., 2010) and flash frozen.

2.5.2 Radioactive in situ hybridization

Sections were fixed in 3% paraformaldehyde, rinsed with 1× phosphate-buffer saline (PBS), dehydrated in ethanol, air dried, and hybridized with 1×10^6 cpm per slide of antisense and sense ^35^S-UTP-labeled riboprobes of the gene of interest. We generated riboprobes from cloned cDNA of mouse Kdm6b (accession # NM_001017426) and mouse Bdnf (accession # NM_001017426). Probes were hybridized at 65°C. After hybridization, slides were dehydrated and exposed to X-ray films (Kodak, USA) for 1–7 days. Slides were then dipped in autoradiographic emulsion (Kodak) and incubated at 4°C for 1–3. They were then processed with D-19 developer (Kodak) and fixer (Kodak), washed, counterstained with cresyl-violet acetate solution (Sigma, USA) and coverslipped with Permount (Sigma). Autoradiographs were visualized by exposure to x-ray film.
2.5.3 Fluorescent in situ hybridization and immunohistochemistry

Fluorescent in situ hybridization was carried out according to published protocols. Briefly, whole brains that were embedded in OCT and flash frozen were cryosectioned at 18um. Digoxigenin (DIG)-labeled riboprobes targeting mouse \textit{Kdm6b} exon III (NM_001017426) were used. Hybridized riboprobes were visualized by fluorescent detection with peroxidase-conjugated anti-DIG Fab fragments (Roche) and developed using TSA fluorescent systems (Perkin Elmer).

\textit{Kdm6b} sense probe 5’- GTCGACCATCGGGCAGTGACCCTC- 3’

\textit{Kdm6b} antisense probe 5’- GGATCCGACCTTGGCTCTGTGAC- 3’

Immunohistochemistry was carried out using standard protocols and using mouse \(\alpha\)-human Ki67 (BD Pharmingen) antibody and 4,6-diamidino-2-phenylindole (DAPI).

2.5.4 Hippocampal culture

Hippocampal neurons from E18 Long Evans rat embryos (Charles River Laboratories) were cultured according to published protocols (Tao et al., 2002). Briefly, the hippocampi were dissected, cells were dissociated with papain (Worthington), and the cell suspension was plated on poly-D-lysine (PDL)-coated dishes in Neurobasal medium with B27 supplements (Invitrogen) and Pen/Strep. Activity-dependent gene transcription was induced using a variety of stimuli. Neurons were depolarized on day 5 \textit{in vitro} (5 DIV) with 55mM KCl in an isotonic
solution (Tao et al., 2002). Cultures were treated on day 5 in vitro (DIV5) with 1uM tetrodotoxin (TTX, Sigma). Cells were treated with TTX for 48 hrs RNA or treated for 48hrs and withdrawn from TTX for different times prior to RNA harvesting. For withdrawal cells were washed twice with plain NB prior to the addition of TTX-lacking conditional media obtained from other wells of untreated neurons. Cells were treated with Bicuculline (Sigma) at 50uM along with 2.5mM 4-AP (Totris) on day 14 in vitro (14DIV) as previously described (Hardingham et al., 2002).

### 2.5.5 RNA harvesting and real time PCR

Seized or control brains were rapidly dissected on ice, hippocampi extracted, snap frozen in liquid nitrogen, and stored at -80°C. Hippocampal cultures were lysed and stored at -80°C. RNA was harvested using the Absolutely RNA kit (Stratagene). 800ngs of RNA were used for reverse transcription with oligo dT primers and Superscript II (Invitrogen). cDNA was used for quantitative PCR of gene transcripts (Power SYBR Green, ABI 7300 real-time PCR machine; Applied Biosystems) with the intron-spanning primers. Each sample was measured in triplicate, and expression of the housekeeping gene Gapdh was used as a normalizing control for RNA quantity and sample processing. All data shown are derived from 2-3 samples.

### 2.5.6 Statistical analysis

Unless otherwise indicated, all data presented are the average of at least
three measurements from each of at least two independent experiments. Also unless otherwise indicated, data were analyzed by a Student’s unpaired t-test, and p<0.05 was considered significant. Bar and line graphs show mean values and all error bars show S.E.M.
3. Kdm6b in the cerebellum

In the second part of the thesis the cerebellum is used as a model system for the study of the histone demethylase Kdm6b in neuronal differentiation. The cerebellum was chosen both because distinct stages in neuronal development are clearly separated in space across the postnatal cerebellum and because large numbers of granule cells can be obtained from the cerebellum, facilitating biochemical studies.

3.1 Summary

As with any other cell type, proper development of neurons requires the regulated expression of specific gene programs. One way to regulate gene expression is via the modulation of histone modifications associated with the regulatory regions of the genes. Kdm6b is a histone demethylase that can remove repressive methyl groups from histones to activate gene expression, and is known to be important for the early stages of neural development. However, its functions in the later stages of development are not known. To fill in this gap in our knowledge, we performed in situ analysis to identify regions of high endogenous Kdm6b expression. We discovered that Kdm6b shows a specific pattern of expression in the inner part of the external granule layer of the cerebellum, which is a region where post-mitotic differentiating neurons are found. We also found that in culture, Kdm6b is important for the survival of CGNs, and is necessary for a gene expression program that is critical for the late differentiation of cerebellar granule neurons. Together these data have helped
us identify heretofore undiscovered functions for Kdm6b in neuronal
development.

3.2 Introduction

Differentiation of specialized cell types from less specialized stem cells or
progenitor cells is critical for the normal formation of various tissue and organ
systems within the body. During development, dynamic regulation of gene
expression patterns is one important step in the process through which
multipotent progenitor cells become mature, terminally differentiated cells.
During such lineage-specification “choices” at the cellular level, modulation of the
chromatin environment is one important way that cell fate-specific gene
expression patterns are established (Mikkelsen et al., 2007). Genes important
for development and cell type-specification that are silent in embryonic stem (ES)
cells are often considered ‘bivalent’ or ‘poised’ in that both silencing (H3K27me3)
and activating (H3K4me1/me3) histone methyl marks are present at promoter
and enhancer regions (Bernstein et al., 2006; Mikkelsen et al., 2007; Rada-
Iglesias et al., 2010). During ES cell differentiation many of these genes lose
their H3K27me3 methyl marks, thereby allowing these genes to be turned “on.”
This change in histone methylation facilitates the differentiation of such ES cells
into specific cell types, such as neuronal cell lineages. Histone demethylases
are known to play an important role in the regulation of histone methylation
during cell fate determination. However, little is known about how histone
methylation in the promoter or enhancer regions of neural-specific genes is
regulated during the later differentiation stages in neuronal cell types.

The methylation state at any specific lysine residue of a histone protein is a result of the balance of the activities of histone methyl transferases (HMTs; the enzymes that can add methyl groups to histones) and histone demethylases (HDMs; the enzymes that can remove methyl groups to histones). Multiple HDMs are associated with neurological diseases such as intellectual disability, epilepsy, autism, and schizophrenia (Laumonnier et al., 2005; Najmabadi et al., 2011; Tahiliani et al., 2007; Tzschach et al., 2006). These data suggest that these HDMs perform crucial functions in the nervous system. One of the HDMs associated with intellectual disability is the H3K27-specific demethylase Kdm6b, which is also known to be important for neuronal lineage establishment (Burgold et al., 2008; Sen et al., 2008). When ES cells differentiate into neural stem cells (NSCs) Kdm6b is required for normal expression of several neuronal genes, including the neural lineage marker Nestin. Kdm6b is recruited to the Nestin promoter, where it removes the repressive H3K27me3 marks to activate Nestin expression (Burgold et al., 2008). During retinoic acid (RA)-mediated differentiation of NSCs into neurons, Kdm6b expression is sufficient to drive the expression of several neuronal genes (Jepsen et al., 2007). Together these studies establish a role of Kdm6b in neuronal lineage establishment. However, whether Kdm6b is required for terminal neuronal differentiation remains unknown.
In order to examine the role of Kdm6b in terminal neuronal differentiation, we decided to use cerebellar granule neuron (CGN) cultures as a model system. The cerebellum is uniquely suited for the study of neuronal development for several reasons. CGNs develop through a series of developmental events that include proliferation, differentiation, migration, and maturation. The cerebellum forms as a strictly layered structure; therefore the developmental stage of a CGN can be easily determined by its location. CGN progenitors form a proliferative zone in the outmost layer of the developing cerebellum (outer external granule layer [EGL]) (Altman and Bayer, 1997). They exit cell cycle and become immature neurons in the inner part of the EGL (inner EGL). They then migrate inwards through the molecular layer to the inner granule layer (IGL) where they mature and become terminally differentiated neurons. CGNs constitute the largest homogenous neuronal population in the mammalian brain. They can be isolated in large numbers and can be cultured in vitro under conditions that recapitulate their in vivo developmental processes. Because CGNs in culture exhibit the same developmentally-regulated gene expression profiles seen in vivo (Okazawa et al., 2009; Sato et al., 2005), this makes them an ideal system for the study of molecular mechanisms that underlie CGN differentiation and maturation.

In this study, we investigated the functions of the H3K27-specific HDM Kdm6b in the regulation of CGN differentiation. By in situ hybridization analysis we identified the robust upregulation of Kdm6b in newborn CGNs. This
development-stage-specific expression of Kdm6b suggested to us that Kdm6b may have precise functions at specific steps of CGN differentiation. We discovered that Kdm6b expression is important for CGN survival and necessary for the expression of a late program of differentiation-related gene expression. These data provide the first evidence for Kdm6b function in post mitotic neurons.

3.3 Results

3.3.1 Kdm6b expression in the cerebellum

3.3.1.1 Kdm6b is expressed in the post-mitotic neurons of the cerebellum

In order to identify the distribution of Kdm6b in the developing cerebellum we carried out in situ hybridization analysis. We discovered that Kdm6b expression in the P7 cerebellum displays an intriguing expression profile. At this age, CGNs of all stages of development are present in the cerebellum: CGN progenitors reside in the outermost subpial region of the cerebellum (outer EGL); immature CGNs, which are post-mitotic and pre-migratory, reside in the layer beneath (inner EGL); migrating CGNs reside in the molecular layer; and mature CGNs reside in the innermost layer (IGL). We saw the highest Kdm6b expression in the inner EGL, less expression in the IGL, and no detectable expression in the outer EGL of the cerebellum (Figure 7). According to this expression profile, Kdm6b is not expressed in the progenitors of the outer EGL, but is robustly upregulated once the CGNs have exited the cell cycle and moved inwards to the inner EGL to become immature neurons.
Figure 7: In situ localization of Kdm6b in the P7 cerebellum. In situ hybridization was done using DIG-labeled riboprobes targeting Kdm6b exon III. Hybridized riboprobes were visualized by immunological detection with alkaline phosphatase-conjugated antibodies.
To further confirm that the upregulation of $Kdm6b$ occurs not in the CGN progenitors of the outer EGL but the post-mitotic neurons of the inner EGL, we stained the cerebellum with Ki67- and Dcx-specific antibodies. Ki67 is a well-established marker of proliferation and stains the dividing CGN progenitors of the outer EGL. Dcx is a well-established marker of immature neurons and stains the post-mitotic immature neurons of the inner EGL. As expected, Ki67 and Dcx stained the outer and the inner EGLs respectively. Co-staining with Ki67 and Dcx show the clear separation of the two layers (Figure 8). We combined fluorescent in situ hybridization for $Kdm6b$ and immunohistochemistry for Ki67 to compare the expression profiles of $Kdm6b$ and Ki67. The lack of overlap between $Kdm6b$ and Ki67 signals confirms that the upregulation of $Kdm6b$ does not occur until the cells have exited the cell cycle and migrated into the inner EGL (Figure 8). This data provides the first evidence for $Kdm6b$ expression in post-mitotic neurons and suggests novel functions for Kdm6b in neuronal differentiation.

3.3.2 Kdm6b in CGN survival

During the development of the cerebellum, CGN progenitors are generated in excess in the EGL. These cells either migrate inwards to the IGL to become mature neurons or are eliminated by apoptosis. The EGL is a site of robust apoptosis of CGNs (Kokubo et al., 2009). We have discovered that $Kdm6b$ shows robust expression in the EGL. We have also discovered that $Kdm6b$ expression is induced by neuronal activity and BDNF in hippocampal
Figure 8: *Kdm6b* is largely excluded from the outer EGL in P7 cerebellum. 

**A.** Immature neuron marker Dcx and proliferating cell marker Ki67 allow for the identification of the inner and the outer EGL, respectively. Immunohistochemistry for Dcx (green) and Ki67 (red). Nuclear stain dapi (blue).

**B.** Fluorescent *in situ* hybridization for *Kdm6b* and immunohistochemistry for Ki67 (green). *Kdm6b* probe hybridization detected with TSA Plus cyanine 3 (red).
neurons, both of which are known prosurvival signals for CGNs. Together, these data led us to hypothesize that Kdm6b could promote the survival of CGNs.

3.3.2.1 **Kdm6b is not necessary for the survival of CGNs in culture**

CGNs provide a robust system for the study of the mechanisms that govern neuronal cell death, and this system has been widely used in studies of neuronal survival (D'Mello et al., 1993; Dudek et al., 1997). Like other neurons in the brain, survival of CGNs during normal development is promoted by growth factors and neuronal activity (D'Mello et al., 1993). The survival of primary CGNs in culture can be supported by polypeptide growth factors and by mimicking the activation of VSCCs by inducing membrane depolarization (Bonni et al., 1999).

To test the requirement for Kdm6b in CGN survival, we first knocked down Kdm6b in CGNs cultured under conditions that support survival – in the presence of growth factors, and under high KCl concentrations that allow membrane depolarization. Cells were isolated from P7 cerebellar, plated on poly-ornithine plated coverslips, and at 3 days *in vitro* (DIV) shRNA constructs carrying either control or Kdm6b KD constructs were introduced to cells by calcium phosphate transfection. Cells were cotransfected with a GFP plasmid to identify transfected neurons. Following transfection, cells were maintained in culture for 2 days and 12, 18, or 24 hours (5DIV + 12,18, or 24 hours) before fixing. Apoptotic cells were identified by their pyktonic nucleai. Under these conditions, we observe less than 10% cell death in both control and Kdm6b knockdown neurons,
demonstrating that *Kdm6b* KD has no effect on survival of CGNs under conditions where the survival is supported by multiple survival promoting factors (data not shown).

### 3.3.2.2 *Kdm6b* is important for growth factor- and activity deprivation-induced neuronal cell death

After discovering that *Kdm6b* is not necessary for the survival of CGNs under conditions that normally promote a high rate of cell survival, we wanted to know whether *Kdm6b* was important for the survival of neurons that were already primed to die. The deprivation of growth factors and neuronal activity, mimicked by the withdrawal of serum and absence of membrane depolarization, potently induces cell death in CGNs (Figure 9). As before, cells were plated on polyornithine coated coverslips in medium containing high KCl and serum and were transfected on day 3 *in vitro* (3DIV) with control or *Kdm6b* KD constructs. Cells were switched to a “minimal medium” containing a low KCl concentration and no growth factors on day 5 *in vitro* (5DIV) and cells were kept in this medium for 12, 18, or 24 hours. As expected, the length of time the cells were kept under these conditions correlated with the amount of cell death that was observed (Figure 9). Consistent with a pro-survival function for Kdm6b, genetic KD of *Kdm6b* led to significantly increased apoptosis at all time points tested as compared to control (Figure 9).
Figure 9: Survival assay. A. Apoptotic cells can be identified by their nuclear morphology (arrows). Cotransfection with GFP allows for the identification of cells with shRNA transfection (arrow heads). B. Time course of CGN cell death upon growth factor withdrawal and activity deprivation. C. KD of Kdm6b increases apoptosis 18hrs after growth factor withdrawal. error bars represent S.E.M. *p < 0.05 compared with control. error bars represent S.E.M., n=3 per group
3.3.2.3 *Kdm6b* is not necessary for BDNF-mediated survival

BDNF and its high affinity receptor TrkB are known to regulate the survival of CGNs in the developing cerebellum *in vivo*, and *Bdnf* KO animals exhibit increased cell death of CGNs (Minichiello and Klein, 1996; Schwartz et al., 1997). Additionally, *in vitro* BDNF can promote survival of CGNs and upon BDNF withdrawal neurons die by apoptosis (Gao et al., 1995; Kubo et al., 1995; Lindholm and Thoenen, 1993; Segal et al., 1995). Neurotrophins like BDNF are known to act through transcription-dependent mechanisms to regulate cellular function (Bonni et al., 1999). Excitingly, we have found that BDNF can drive the expression of *Kdm6b* in cultured CGNs (data not shown). Based on these observations, we hypothesized that Kdm6b might be necessary for BDNF-mediated cell survival of CGNs.

To test this hypothesis, we again used the above-mentioned CGN culture system with modifications. Following 5 DIV, we switched the CGNs to minimal media +/- exogenous BDNF for 12, 18, or 24 hours. Consistent with its already-defined role in cell survival, BDNF was able to partially rescue the cell death phenotype in CGNs kept in minimal medium (Figure 10). Surprisingly, the ability of BDNF to promote the survival of CGNs kept in minimal media was not affected by *Kdm6b* KD, demonstrating that *Kdm6b* is not necessary for the BDNF-mediated survival of CGNs (Figure10).
Figure 10: BDNF-mediated survival of CGNs. A. Addition of BDNF (50ng/ml) alone to minimal medium is sufficient to partially rescue survival of CGNs. CGNs were plated in the presence of growth factors, and high KCl concentrations. At 3DIV cells were transfected with control plasmid, and GFP plasmid to identify transfected cells. On 5DIV cells were switched to the same media or “minimal media” +/- BDNF for indicated times. B. Kdm6b is not necessary for the survival effects of BDNF in CGNs. Cells were transfected with either control or of Kdm6b KD construct.
3.3.3 Kdm6b in CGN differentiation

CGNs in the EGL that survive proceed to differentiate into mature neurons. The differentiation of neuronal progenitors into fully mature neurons is a multistep process that is orchestrated by progressive changes in specific gene expression programs. H3K27 methylation-dependent repression of the Polycomb group of differentiation-specific genes can be overcome by Kdm6b demethylase activity. In differentiating macrophages, Kdm6b function removes repressive H3K37me3 from a cluster of late Hox genes, thereby inducing their expression (De Santa et al., 2007). In the developing cerebellum, we have provided evidence that Kdm6b is expressed in cells that are differentiating towards a mature phenotype. This observation, combined with the fact that Kdm6b is important for the differentiation of macrophages, led us to hypothesize that Kdm6b may be important for the regulation of gene expression programs that drive CGN differentiation.

3.3.3.1 shRNA-mediated knockdown (KD) of Kdm6b in CGN progenitors

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3.3.3.2 Kdm6b regulates a subset of maturation genes in CGNs

Due to the huge abundance of CGNs in the cerebellum as compared with other cell types, RNA isolated from whole cerebella of different ages has been used to identify developmentally-regulated gene expression patterns that might be important for CGN differentiation (Sato et al., 2005). Given evidence from previous studies that primary cultures of purified CGN progenitors recapitulate many properties characteristic of differentiating CGNs in vivo (Mellor et al., 2000; Suzuki et al., 2005), we decided to examine the temporal expression profiles of several developmentally-regulated genes in our CGN progenitor cultures (Figure 11). We purified CGNs and maintained them in culture as described in the previous section, and examined gene expression by real time-PCR. Because we were not interested in delaying the onset of differentiation in this experiment, we did not add Shh to the cultures at any point. Known marker genes for individual stages of CGN development showed differential regulation over developmental time, consistent with what has been previously demonstrated (Díaz et al., 2002).
Figure 11: Differentiating CGNs in culture recapitulate their *in vivo* gene expression profiles. *Math1* is expressed in progenitors and its expression drastically decreases as the progenitors rapidly differentiate into immature neurons upon plating. *Gabra6* is highly expressed in the mature CGNs of the IGL and its expression increases as cells mature. Switch of NMDA receptor subunits mark a milestone in CGN development *in vivo*. *Grin2b* is highly expressed in the CGNs of the EGL while *Grin2c* is highly expressed in the CGNs of the IGL. In culture, *Grin2b* expression decreases as the CGNs mature, while *Grin2c* expressed increases. CGN progenitors were isolated from P7 cerebellar and cultured for 0, 3, 7, 10, or 14 days. RNA was isolated, cDNA synthesized, and SYBR green PCR used to quantify gene expression. All data were normalized to expression of the housekeeping gene *Gapdh* as control for sample handling. Data are expressed as fold induction over day 0 expression. n=3
Math1, an early marker expressed during the specification of CGN precursors, shows downregulation as CGNs differentiate (Ben-Arie et al., 1997), while the expression of Gabra6 (the gene that encodes GABA receptor α6 subunit), a marker of mature CGNs in the IGL (Bahn et al., 1997), increases as CGNs differentiate in culture. Furthermore, consistent with an important developmental switch in NMDA receptor subunit composition known to occur \textit{in vivo}, Grin2b expression decreases and Grin2c expression increases (Iijima et al., 2008). \textit{In vivo}, immature pre-migratory CGNs of the inner EGL express Grin2b. As the cells differentiate and migrate into the IGL, Grin2b expression is downregulated and Grin2C expression is upregulated. Taken together, these expression profiles confirmed to us that in our culture system, CGNs differentiate as expected.

To determine whether Kdm6b plays a role in CGN differentiation via regulation of gene expression, we assessed the expression levels of several of the developmentally-regulated genes identified by Sato and colleagues (2005) as belonging to a late CGN differentiation program under control and \textit{Kdm6b} KD conditions. Control and \textit{Kdm6b} KD cells were cultured in the presence of Shh for 3 days and then cultured in differentiation media for 2 or 6 days (Figure 12). Following 2 or 6 days in differentiation media, RNA was isolated, cDNA synthesized, and real-time PCR carried out using primers specific to the genes of interest. Under control infected conditions, the genes tested recapitulate the expression patterns seen \textit{in vivo} (Sato et al., 2005) and in our cultures \textit{in vitro} if CGNs were cultured directly in neuronal media following isolation (Figure 13).
Figure 12: Successful KD of Kdm6b in CGN progenitors. A. Cerebellar granule neuron (CGN) progenitors were isolated from P4 mouse cerebella and cultured for 3 days in the presence of Shh to maintain the mitotic progenitor state. Control or Kdm6b knockdown (KD) virus was added at the time of plating. At the end of 3 days, the cell aggregates that formed in Shh-containing media were transferred to Shh-lacking, neuronal (differentiation) media and cultured for 2 days or 6 days before harvesting. B. RNA was isolated, cDNA synthesized, and SYBR green PCR run using Kdm6b-specific primers to quantify gene expression. All data were normalized to expression of the housekeeping gene Gapdh as control for sample handling. Data are expressed as fold induction over control day 2 expression. n=1-2
Figure 13: Expression profiles of several developmentally regulated genes can be recapitulated in vitro. CGN progenitors were isolated and cultured for 3 days in the presence of Shh. Cells were transferred to neuronal (differentiation) media and cultured for 2 days or 6 days. RNA was isolated, cDNA synthesized, and SYBR green PCR used to quantify gene expression. All data were normalized to expression of the housekeeping gene Gapdh as control for sample handling. Data are expressed as fold induction over day 2 expression. n=1-2
The expression levels of developmentally upregulated genes *Grin2c, Grin2a, Gabra1, and Gabra6 and Tiam-1* increase from day 2 to day 6, while the expression levels of the developmentally downregulated genes *Dcx* and *Grin2b* decrease from day 2 to day 6. These results confirmed to us that the pre-incubation in Shh does not disrupt the differentiation of CGNs.

Under *Kdm6b* KD conditions, however, the expression of the developmentally upregulated genes *Grin2c, Grin2a, Gabra1, and Gabra6* was significantly reduced (Figure 14). On the contrary, *Kdm6b* KD has no effect on the expression levels of the developmentally downregulated genes *Dcx* and *Grin2b*, or of *Tubb*, a gene that is not developmentally regulated in CGNs (Figure 15). Kdm6b is a histone demethylase that removes repressive H3K27me3 histone marks to activate gene expression. The lack of an effect on the expression patterns of downregulated or non-developmentally regulated genes indicates that *Kdm6b* KD might specifically alter the expression of genes whose induction is dependent on Kdm6b’s demethylase enzymatic activity, and suggests that loss of *Kdm6b* does not have a more global, non-specific effect on gene expression.

Jepsen and colleagues (2007) reported that overexpression (OE) of *Kdm6b* in cultured NSCs is sufficient to drive *Dcx* expression. This suggests that *Dcx* could be a target gene of Kdm6b. *Kdm6b* and *Dcx* are indeed expressed in the same cells at the same time in the developing cerebellum; they are both prominently expressed in the inner EGL and their expression persists, albeit to a
Figure 14: *Kdm6b* is necessary for the upregulation of a battery of late developmental genes. CGN progenitors were isolated and cultured for 3 days in the presence of Shh and in the presence of either control or KD virus. Cells were transferred to neuronal (differentiation) media and cultured for 2 days or 6 days. RNA was isolated, cDNA synthesized, and SYBR green PCR used to quantify gene expression. All data were normalized to expression of the housekeeping gene Gapdh as control for sample handling. Data are expressed as fold induction over day2 expression. n=1-2
Figure 15: *Kdm6b* is not necessary for the developmental downregulation of gene expression. CGN progenitors were isolated and cultured for 3 days in the presence of Shh and in the presence of either control or KD virus. Cells were transferred to neuronal (differentiation) media and cultured for 2 days or 6 days. RNA was isolated, cDNA synthesized, and SYBR green PCR used to quantify gene expression. A-B. The developmental downregulation of *Grin2b* and *Dcx* are not affected by *Kdm6b* KD. C. *Kdm6b* KD does not affect expression levels of *Tubb5*. All data were normalized to expression of the housekeeping gene *Gapdh* as control for sample handling. Data are expressed as fold induction over day2 expression. n=1-2
lesser level, to the IGL (Sato et al., 2005). However, based on our data, the lack of alteration of $Dcx$ expression under $Kdm6b$ KD conditions appears to eliminate the possibility that Kdm6b is necessary for $Dcx$ expression. The observation by Jepsen and others that $Kdm6b$ overexpression is sufficient to drive $Dcx$ expression could be a result of the OE conditions. For example, it is possible that under OE conditions Kdm6b is recruited to promoters that are not usually targets of Kdm6b. $Dcx$ could also have a low threshold requirement for Kdm6b function, such that the small percentage of $Kdm6b$ the might be remaining under our KD conditions could be sufficient to drive normal $Dcx$ expression, even when we achieve significant KD of $Kdm6b$, and when we introduce shRNA-carrying viruses to progenitor cells and maintain them in the progenitor state for 3 days to ensure that the RNAi machinery is well underway by the time differentiation occurs.

3.3.3.3 $Kdm6b$ is required for BDNF-induced gene expression

Sato and colleagues discovered that BDNF can drive the expression of several late differentiation program genes that are important for the proper maturation and function of CGNs (Sato et al., 2006). To test the importance of Kdm6b in the BDNF-induced expression of these genes, we cultured control or $Kdm6b$ KD cells with or without BDNF. Following 3 days of culture in the presence of Shh, control and $Kdm6b$ KD cells were moved to differentiation media +/- BDNF. Cells were maintained in the differentiation media +/- BDNF for 2 days or 6 days, at which time RNA was isolated, cDNA synthesized, and SYBR
green real-time PCR carried out to quantify expression levels. We tested two genes identified by Sato and colleagues as BDNF-dependent genes. As expected, in control cells both genes tested, *Grin2C* and *Tiam1*, showed enhanced expression when cultured in the presence of BDNF. In contrast, this BDNF-mediated enhancement of expression does not occur under *Kdm6b* KD conditions, suggesting a requirement for *Kdm6b* function in the BDNF-dependent regulation of these genes (Figure 16).

3.4 Discussion

Published reports of *Kdm6b* functions in the nervous system are limited to very early developmental events. In ES cells, *Kdm6b* is required for the establishment of the neural lineage, and in NSCs *Kdm6b* is sufficient to drive the expression of several neuronal genes (Burgold et al., 2008; Jepsen et al., 2007). In the chick embryo, *Kdm6b* is important for the establishment of dorsal interneurons from neuroepithelial precursor cells (Akizu et al., 2010). Our *in situ* hybridization data (combined with immunohistochemistry) clearly identify the expression of *Kdm6b* in post-mitotic differentiating neurons of the cerebellum. We have also identified functions for *Kdm6b* in these cells in neuronal survival and the expression of a late stage differentiation gene program. A role for *Kdm6b* in cell differentiation has also been reported in non-neural cells; *Kdm6b* drives expression of differentiation genes in macrophages and the epidermis (De Santa et al., 2007; Sen et al., 2008). Together, these studies suggest context-
Figure 16: *Kdm6b* is permissive for BDNF-driven enhancement of gene expression. **A.** CGN progenitors were isolated and cultured for 3 days in the presence of Shh and in the presence of either control or KD virus. Cells were transferred to neuronal (differentiation) media +/- BDNF and cultured for 2 days or 6 days. RNA was isolated, cDNA synthesized, and SYBR green PCR used to quantify gene expression. **B.** Control cells cultured in differentiation media in the presence or absence of BDNF. *Grin2c* expression levels are higher in cells grown in the presence of BDNF compared to ones grown without BDNF. **C.** Control and *Kdm6b* KD cells were cultured in the presence of BDNF in differentiation media. BDNF cannot enhance the expression *Grin2c* under *Kdm6b* conditions. All data were normalized to expression of the housekeeping gene *Gapdh* as control for sample handling. Data are expressed as fold induction over day2 expression. n=2
dependent roles for Kdm6b in multiple cell types and a common function in promoting differentiation across different cell types. Presumably, Kdm6b is specifically recruited by different cofactors to different genes in different cell types at particular stages of development in order to promote these diverse functions. Some of the co-factors Kdm6b interact with in order to be recruited to DNA regulatory elements are known. In the embryonic dorsal nerve cord, in response to BMP signaling, Kdm6b interacts with Smad proteins to be recruited to target genes (Akizu et al., 2010; Dahle et al., 2010). In lymphocytes, Kdm6b is recruited to target genes by the transcription factor T-bet (Miller et al., 2010). Finally, in a breast cancer cell line, Kdm6b associates with ERα in a ligand-dependent manner to be targeted to enhancer regions (Svotelis et al., 2011). Future studies will be required to identify the cofactors that mediate Kdm6b recruitment to target promoters in the cerebellum.

3.4.1 Kdm6b in CGN Survival

We have demonstrated that expression of Kdm6b is important for the survival of cultured CGNs. Consistent with this finding, in hippocampal neurons, Kdm6b is activated by stimuli that are known to promote survival – synaptic NMDA receptor activation and BDNF – suggesting that Kdm6b may have common functions in promoting survival in different neuronal cell types. Kdm6b has been shown to be important for survival in a non-neural system as well. In a breast cancer cell line the depletion of Kdm6b resulted in increased apoptosis (Svotelis et al., 2011), suggesting that the pro-survival function of Kdm6b is not
restricted to neurons. As a HDM that removes repressive methyl marks, Kdm6b probably promotes survival by activating the expression of pro-survival genes. However, we have yet to identify the genes that are targeted by Kdm6b to promote survival in CGNs. One candidate gene is the anti-apoptotic gene Bcl-2; it is known that the survival of CGNs in culture depends on the expression of the Bcl-2 (Bonni et al., 1999), and Kdm6b is necessary for the expression of Bcl-2 in breast cancer cells (Svotelis et al., 2011), raising the possibility that Bcl-2 is a target gene of Kdm6b in CGNs and that survival is promoted by its Kdm6b-dependent expression.

Since BDNF is known to promote CGN survival (Bonni et al., 1999; Lindholm and Thoenen, 1993), we tested the requirement for Kdm6b in BDNF-mediated rescue of CGNs that had been artificially prompted to undergo apoptosis. We discovered that Kdm6b is not necessary for the BDNF-mediated survival of CGNs. However, BDNF activates multiple signaling cascades and promotes survival through transcription-dependent and -independent mechanisms in CGNs (Bonni et al., 1999). The fact that BDNF can promote survival even in the absence of Kdm6b suggests that Kdm6b is not at a fundamental/convergent point of the pro-survival pathways that are activated by BDNF, but does not eliminate the possibility that Kdm6b is involved in one or more (but not all) of the signaling pathways activated by BDNF. NF-KB, a transcription factor that mediates Kdm6b expression in macrophages, is important for the survival effects mediated by IGF (Koulich et al., 2001); it will be
interesting in the future to see whether Kdm6b is necessary for the IGF-mediated survival in CGNs.

**3.4.2 Kdm6b in CGN differentiation**

Based on its expression profile in the developing cerebellum, we hypothesized that Kdm6b may have roles in CGN differentiation and maturation. We identified that *Kdm6b* expression is required for the expression of a late differentiation gene program in cultured CGNs (Abe et al., 2011). Some potential Kdm6b target genes, including *Grin2c*, *Gabra6*, and *Gabra1*, encode proteins that function as receptor subunits. The coordinated regulation of these receptor proteins is essential for functional synaptic transmission and synapse maturation in the cerebellar circuit (Brickley et al., 2001). *Grin2c* upregulation is necessary for the establishment of an important milestone in cerebellar development: during cerebellar development, the subunit composition of NMDA receptors switches from NR2B-subunit containing receptors to NR2C-subunit containing receptors. This switch alters many properties of the NMDA receptors and is important for the establishment of mature mossy fiber–granule cell synaptic transmission (Nakanishi, 2005). GABA\(\alpha\)1 and \(\alpha\)6 subunits are important for the formation of GABAergic synapses between CGNs and golgi cells and for functional inhibitory synaptic transmission (Takayama and Inoue, 2004). The inhibitory synapses between golgi cells and CGNs suppress the excitation of CGNs by mossy fiber inputs, ultimately modulating the neuronal activity of Purkinje cells, which are the major cerebellar cortical output. Mice lacking any one of these genes appear
largely normal under standard conditions (e.g., Grinc KO mice or Gabra6 KO mice), but can show alterations in drug sensitivity or synaptic activity, as is the case with Gabra6 KO mice (Mellor et al., 2000). The potential Kdm6b target gene Tiam-1 encodes a Rac1-specific guanine nucleotide exchanging factor that can interact with NMDA receptors to mediate their effects on dendritic and spine development (Minard et al., 2004). Tiam-1 is expressed in the migrating CGNs of the molecular layer and is implicated in neuronal migration (Ehler et al., 1997; Sato et al., 2005). Since Kdm6b is necessary for the expression of multiple genes, including Tiam-1, whose gene products are important for the formation and function of CGN synapses, it is reasonable to assume that in the absence of Kdm6b the formation and function of CGN synapses would be disrupted. Due to the homogeneity of the in vitro culture system, we cannot test the formation and function of synapses. However, this issue could be explored in slice cultures or in Kdm6b conditional KO mice (constitutive KO of Kdm6b dies perinatally before the onset of CGN differentiation in the cerebellum), which would also allow for assessment of behavioral changes due to the lack of Kdm6b.

Based on the H3K27-specific HDM activity of Kdm6b, our model for activation of gene expression by Kdm6b is the removal of the repressive H3K27me3 methyl mark from the regulatory regions of target promoters following Kdm6b recruitment to these promoters. However, there are several examples that challenge the demethylase activity-dependent gene activation model for Kdm6b. In differentiating NSCs, Kdm6b overexpression is sufficient to induce
the expression of Arf. It has been suggested that the ARF-p53 pathway attenuates self-renewal and promotes differentiation of NSCs (Nagao et al., 2008), and the PcG gene Bmi1 promotes cell proliferation and neural stem cell self-renewal by repressing the INK4a/ARF locus (Bruggeman et al., 2005). During NSC differentiation, Kdm6b binds to Arf promoter, but there are no detectable changes in H3K27me3 levels to parallel Kdm6b binding and the changes in expression ((Solá et al., 2011). In another example in macrophages, the regulatory regions bound by Kdm6b and associated with genes whose expression is decreased following Kdm6b KD do not show alterations in H3K27me3 levels during activation or following Kdm6b KD. Furthermore, Kdm6b but not its demethylase activity is necessary in lymphocytes for the recruitment of the SWI/SNF chromatin remodeling complex to target promoters (Kdm6 itself is recruited to the promoter by the transcription factor T-bet) (Miller et al., 2010). These studies suggest that, in at least some cases, Kdm6b has demethylase-independent functions for modifying chromatin architecture and gene expression.

While we did not find that Kdm6b is required for BDNF-mediated CGN survival, our data showing that BDNF-induced upregulation of Grin2c and Tiam-1 expression is impaired by Kdm6b KD suggests that Kdm6b may be required for other BDNF-dependent processes in the cerebellum, such as CGN differentiation and synapse maturation. However, it is difficult to determine whether this requirement is due to Kdm6b acting as a direct downstream mediator of BDNF
signaling or whether Kdm6b is simply de-repressing the promoters (and/or other regulatory regions) to create a permissive environment for transcription. Given that we already see diminished baseline expression of these genes under Kdm6b KD conditions, it is possible that in the absence of Kdm6b activity the repressive H3K27me3 mark persists in the target promoters and therefore the genes cannot be activated even in the presence of BDNF signaling. Thus, it is difficult to ascertain whether Kdm6b directly mediates BDNF signaling, or plays a permissive role, or both. However, it is clear that Kdm6b function is necessary for BDNF-induced expression of Grin2c and Tiam-1 regardless whether Kdm6b is a direct transducer of BDNF signaling or not.

Another possible reason for the lack of BDNF-mediated enhancement of expression of Grin2c and Tiam-1 in Kdm6b KD cells is that BDNF-dependent signaling cascades might be impaired under Kdm6b KD conditions. Although this scenario is unlikely, we wanted to rule it out by confirming that the BDNF signaling pathway was intact under Kdm6b KD conditions. There are several genes whose expression is downregulated in the presence of BDNF in CGNs (Sato et al., 2006). To confirm that BDNF signaling is intact under Kdm6b KD conditions, we looked at the expression of genes known to be downregulated in expression by BDNF signaling. Given that we have shown evidence that Kdm6b is important for gene activation, but not gene inactivation, we predicted that Kdm6b KD should not affect the expression of genes that are downregulated in
response to BDNF signaling. *Ptprk* and *Bahl1* are developmentally
downregulated genes whose expression levels are further decreased in the
presence of BDNF. However, in our hands, *Ptprk* and *Bahl1* did not show the
developmental- or BDNF-mediated-regulation that has been reported (data not
shown). Thus, further work will be needed to address this question.

### 3.5 Experimental methods

#### 3.5.1 Animals

C57BL/6 mice were bred and maintained in the animal facility at Duke
University under a 12 h light (6 A.M. – 6 P.M.), 12 h dark (6 P.M – 6 A. M.) cycle.
Food and water were provided *ad libitum* and all care was given in compliance
within National Institute of Health and Duke University institutional guidelines on
the care and use of laboratory and experimental animals under Institutional
Animal Care and Use Committee approved protocol.

#### 3.5.2 *In situ* hybridization and immunohistochemistry

For *in situ* hybridization analysis and immunohistochemistry, whole brains
were harvested from 7-day-old (P7) mice, flash frozen, embedded in OCT, and
cryosectioned at 18um. *In situ* hybridization was carried out according to
standard protocols (Welch). Digoxigenin (DIG)-labeled riboprobes targeting
mouse *Kdm6b* exon III (NM_001017426) were used. Hybridized riboprobes were
visualized by immunological detection with alkaline phosphatase (AP)-conjugated
anti-DIG antibodies (Roche) and developed using 5-brom-4-cloro-
indolylphosphate/nitroblue tetrazolium (BCIP/NBT; Roche) or by fluorescent detection with peroxidase-conjugated anti-DIG Fab fragments (Roche) and developed using TSA fluorescent systems (Perkin Elmer).

\[ Kdm6b \text{ sense probe 5'} - \text{GTCGACCATCGGGCAGTGACCCTC} - 3' \]

\[ Kdm6b \text{ antisense probe 5'} - \text{GGATCCGACCTTGGCTCTGTGCTGAC} - 3' \]

Immunohistochemistry was carried out using standard protocols and using the following antibodies and reagents: mouse α-human Ki67 (BD Pharmingen) and goat α-human Dcx (Santa Cruz) antibodies and 4,6-diamidino-2-phenylindole (DAPI).

### 3.5.3 Primary cerebellar granule cell isolation and fractionation

Cerebellar from P7 mice were aseptically removed, cut into small pieces, and incubated at 37°C for 30 mins in digestion buffer consisting of Dulbecco’s PBS (DPBS, Invitrogen) with 10 U/ml papain (Worthington), 200 ug/ml L-cysteine (Sigma), and 250 U/ml DNAse (Sigma). Following incubation, digestion buffer was replaced with DPBS containing 8 mg/ml soybean trypsin inhibitor (Sigma), 8 mg/ml bovine serum albumin (BSA, Sigma), and 250 U/ml DNAse, and the tissue was triturated using pipettes to obtain a single-cell suspension. Cells were centrifuged at room temperature and resuspended in DPBS containing 200 ug/ml BSA. In order to enrich for CGN progenitors, the cell suspension was underplayed with a step gradient of 35% and 65% Percoll (Sigma) and centrifuged at 2500 rpm for 12 min at room temperature. CGN progenitors were harvested from the 35/65% interface, washed in DPBS/BSA, resuspended in
Neurobasal medium (Gibco/Invitrogen) supplemented with B27 supplement (Gibco), sodium pyruvate (1mM) (Invitrogen), L-glutamine (2mM) (Gibco), and penicillin/streptomycin (Gibco). Cells were transferred to tissue culture dishes coated with poly-D-lysine (PDL)/laminine and incubated in a 95%O<sub>2</sub>/5%CO<sub>2</sub> atmosphere at 37°C.

When preparing cells for survival assay, Percoll gradient step was avoided. Following DPBS/BSA wash CGNs were resuspended in BME (Gibco) containing calf serum (HyClone) and membrane-polarizing concentrations of KCl (25mM) and cultured on German glass coverslips (Bellco; catalog # 1943-10015).

### 3.5.4 Calcium phosphate transfection

CGNs on glass coverslips were transfected by calcium phosphate method (Tao et al., 2002) after 2 days in vitro (P7 + 2DIV) with Kdm6b KD or control plasmid together with a plasmid encoding GFP.

### 3.5.5 Survival Assay

After 5DIV, cells on coverslips were washed and returned to conditioned medium or placed in medium deprived of serum and membrane depolarizing concentrations of KCl (5mM) for 18, 24, or 36hrs. 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 bisbenzimide (Hoechst 33258; Sigma). Cell counts were
carried out blind and analyzed by student’s unpaired t-test. Approximately 150 cells from 3 coverslips were counted per experiment.

### 3.5.6 Lentivirus infection of CGN progenitors

For viral infection of neurons, Kdm6b shRNA constructs were packaged as lentivirus particles in HEK 293T cells (ATCC, Manassas, VA). CGN progenitors were infected with virus following the protocol of Zhou et al. 2007 with modifications (Zhou et al., 2007). 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 0.4ug/ml poly-brene (Sigma) and 4.5 ug/ml Shh-N 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 or 4 days prior to processing for RNA. Infection efficiency of CGN progenitors with lentivirus is 80% on average, determined by the percentage of GFP-positive cells.

### 3.5.7 Plasmids

Two independent shRNAs were used to knockdown mouse Kdm6b (NM_001017426). Both shRNAs were purchased from Open Biosystems. The empty pLKO.1 vector was used as the control for both shRNAs.

shRNA1 hairpin sequence -

CGGCCTCTGTCTTTGAGGGACAAAACTCGAGTTTTGTCCCTCAAGAACAGAGGTTTTTG
shRNA2 hairpin sequence –
CCGGCCTGTTCGTTACAAGTGAGAACTCGAGTTCTCACTTTGTAACGAAACAGGTTTTTG

3.5.8 Shh and thymidine-incorporation assay
Recombinant Shh-N was generated in E. coli by using a GST-Shh-N plasmid (Fogarty et al., 2007). Shh supernatant was generated by transfecting 293T cells with Shh-N-expressing plasmid and harvesting supernatant for 3 days. To test the efficacy of Shh-N, proliferation assay was carried out. GCPs were cultured in PDL-coated 96-well plates at 2 x 10^5 cells per well. Shh-N or EGFP (control) was added at the beginning of culture, and cells were incubated for 48 hrs before pulsing with methyl ^3H-thymidine (GE Healthcare, Piscataway, NJ). After 18 hrs, cells were harvested by using a Mach IIIM Manual Harvester 96 (TOMTEC, Hamden, CT), and incorporated radioactivity was quantified using a Wallac MicroBeta microplate scintillation counter (PerkinElmer, Fremont, CA).

3.5.9 RNA extraction and real time PCR
RNA was isolated using a Stratagene Kit according to manufacturer’s directions and quantified spectrophotometrically. Single-stranded cDNA was synthesized using SuperScript II RNAse H Reverse Transcriptase (Invitrogen) according to the manufacturer’s directions. All samples were subjected to 40 cycles of Real Time PCR using PowerSYBR green detection on an ABI7300 (ABI, Foster City, CA). Intron spanning primers were used at all possible times for all genes tested: Kdm6b, 5’-accaccatcgctaaatacgc-3’ (forward) and 5’-ctctgtagctgtgcttc-3’ (reverse), Grin2C 5’- -3’ (forward) and 5’- -3’ (reverse),
Grin2B 5’-gagcataatcaccgcagct-3’ (forward) and 5’-aaggacgtggtcccgttat-3’ (reverse), Gabra1 5’-cccaatgacccgcaagact-3’ (forward) and 5’-aaacgtgccatctctcttgcc-3’ (reverse), Dcx 5’-tcgtagtttgtatgcggtgc -3’ (forward) and 5’-tgcaatatatgtctcagaact-3’ (reverse), Math1 5’-aatgaccaccatcaccttgc-3’ (forward) and 5’-tgctggatctgaggatgtt-3’ (reverse), gapdh 5’-catggcctccggtcgtct-3’ (forward) and 5’-gatggtcattggtgatct-3’ (reverse), and Tiam-1 5’-caacagctccctgttagga-3’ (forward) and 5’-gagttggtgcatcattct-3’ (reverse).

Primer efficiency was tested within each experiment, and product quantities were calculated from a standard curve. Transcript levels were normalized for RNA from Gapdh as control for sample preparation and handling.

### 3.5.10 Statistical analysis

Unless otherwise indicated, all data presented are the average of at least three measurements from each of at least two independent experiments. Also unless otherwise indicated, data were analyzed by a Student’s unpaired t-test, and p<0.05 was considered significant. Bar and line graphs show mean values and all error bars show S.E.M.
4. Discussion

In this thesis I have investigated the expression and functions of the H3K27-specific histone demethylase Kdm6b. Whereas previous studies have focused on Kdm6b in early stages of neuronal fate determination, my studies are the first to show that expression of Kdm6b is robustly regulated by seizure-induced neuronal activity in post-mitotic cells. In addition, my work in CGNs is the first to demonstrate roles for Kdm6b both in neuronal survival and in the expression of mature neuronal differentiation genes. Taken together these data reveal that Kdm6b is an important modulator of neuronal development and function.

4.1 Methylation in activity-dependent gene expression

Seizures drastically increase neuronal activity across many regions in the brain, and several studies have demonstrated that this can stimulate the expression of numerous activity-dependent genes. This is partly achieved by alterations in histone post-translational modifications at the regulatory regions of the activity-responsive genes. The majority of published studies examining the chromatin regulation of gene expression after seizure have focused on changes in histone acetylation and phosphorylation (Tsankova et al., 2004). In our studies, we have discovered robust upregulation of Kdm6b and three other histone demethylases in the post-mitotic neurons of the DG following seizure. Although the potential target genes of these histone demethylases remain
unknown, the robust upregulation in \textit{Kdm6b} expression that we observe following seizure suggests to us that \textit{Kdm6b} might be functioning in these neurons to increase gene expression via its demethylase activity, and thereby facilitating the transduction of neuronal activity into increased gene expression.

Among the IEGs activated by seizure, \textit{Bdnf} is a good candidate gene to explore changes in histone methylation that accompany gene expression because it is one of the best-studied activity-inducible genes. A previous study has shown that following membrane depolarization, which drives \textit{Bdnf} expression, there is a concomitant decrease in H3K9me3 on the \textit{Bdnf} promoter (Chen et al., 2003). Interestingly, in our screen for seizure-induced expression of HDMs we identified several known H3K9-specific demethylases that show upregulation. Together these data suggest that removing the repressive H3K9 methyl marks by upregulating the expression of demethylases specific to this mark could represent one of the mechanisms through which gene expression can be upregulated following membrane depolarization. It should be noted that in a recent genome-wide ChIP-Seq study, very few changes in H3K27me3 were observed in cultured hippocampal neurons following membrane depolarization (Kim et al., 2010). However, our \textit{in vitro} data reveal that \textit{Kdm6b} is not induced in response to this stimulus. Similar genome-wide studies have not been performed in the hippocampus \textit{in vivo} after seizure. However, at least one study did report \textit{in vivo}, stimulus-dependent changes in H3K27me3 on a \textit{Bdnf} promoter (promoter III). In that study, following an animal model of depression called
social defeat stress, reduced expression of *Bdnf* was observed. This was accompanied by an increase in H3K27me3 repressive methyl marks on *Bdnf* promoter III (Tsankova et al., 2006). These data are consistent with the possibility that H3K27me3 of *Bdnf* promoters could regulate *Bdnf* expression in response to extracellular stimuli that alter neuronal activity levels. Our data suggest that pilocarpine-induced seizure *in vivo* may be an excellent stimulus to identify genes that require demethylation of H3K27me3 for their induction.

### 4.2 Potential biological functions of Kdm6b in the cerebellum

Previous loss-of-function studies have shown that Kdm6b is important in early stages of neuronal fate determination. However, all of the studies in my thesis have pointed towards roles for Kdm6b in post-mitotic neurons. Although *Kdm6b* knockout mice have been generated, very little has been reported about neuronal differentiation or plasticity in these mice because they die perinatally. By contrast, I used inducible expression of *Kdm6b* in the adult brain and temporally restricted delivery of RNAi against *Kdm6b* to suggest its role in differentiating neurons. Thus, consistent with the idea that chromatin regulation is important at many stages in development, these data expand the time frame over which Kdm6b is important.

Interestingly, SNPs in *Kdm6b* were recently identified in a screen for genetic causes of intellectual disability (Najmabadi et al., 2011). The SNP in *Kdm6b* is not within the enzymatic domain of the protein, raising the question of how or whether it changes Kdm6b function. It is also not clear whether this...
mutation would affect Kdm6b in all stages of neuronal development. In our study, we have identified that \textit{Kdm6b} is necessary for the expression of a late differentiation gene program in CGNs. Some of these target genes encode proteins that are neurotransmitter receptor subunits that confer mature properties upon synaptic transmission. Lack or depletion of Kdm6b activity therefore could lead to defects in the formation, maturation, or function of synapses. This could then lead to neurological defects and behavioral abnormalities. Importantly, using the CGN differentiation assay I have established, we are now in an excellent position to test whether or not expression of a version of Kdm6b bearing the human mutation associated with intellectual disability can rescue gene expression in the \textit{Kdm6b} knockdown neurons.

How is it that Kdm6b can regulate both early and late stages of neuronal differentiation? It appears that Kdm6b function can be very context-dependent. For example, the specific effects of Kdm6b could be determined by cell type-specific DNA-binding factors and/or cofactors that interact with Kdm6b. Several proteins that interact with Kdm6b such as the T-box binding protein T-bet are already known, although Kdm6b interactors in cerebellar and hippocampal neurons remain to be identified. Identifying repressors and co-repressors that interact with Kdm6b to recruit it to target regulatory regions would help us understand how specificity of Kdm6b function is achieved.

While we have studied Kdm6b for its function as a histone demethylase, it is important to note that the so-called “histone-modifying enzymes” can also have
other roles in the cell beyond histone modification. Specifically, Kdm6b could also modify other proteins in the cell, although there is no record to-date of HDMs acting on non-histone proteins in the nervous system. Interestingly, Kdm6b has been detected on promoters that do not undergo changes in H3K27 methylation levels following its binding (De Santa et al., 2009; Solá et al., 2011). However, in some of these studies, like the one done in macrophages, it is not clear whether the study was done at a point in time when Kdm6b would have been functioning to affect gene expression. There were very few alterations observed not only in H3K27me3 levels, but also in the expression of target genes in Kdm6b KO cells compared to wild type cells. At instances when Kdm6b is not acting as a demethylase, it could still be acting to modify chromatin architecture. At least in one case, Kdm6b has been reported to interact with chromatin remodeling complexes (Miller et al., 2010), and it is possible that this interaction could regulate chromatin structure in a way that would affect gene expression. Kdm6b is a large protein of 180 kDa and it is also possible that it could act as a scaffolding protein to bring different regulatory proteins together. Lastly, Kdm6b could also mediate chromatin looping – a feature in the chromatin architecture that is brought about by the interaction between proteins bound to distant regulatory elements, such as promoters and enhancers – which is highly speculated to play a role in the regulation of gene expression. Although some studies suggest that Kdm6b plays demethylase-independent roles in some systems, Kdm6b still regulates the expression of multiple genes via its
demethylase activity and needs to be appreciated for its function as a histone demethylase.

4.3 Kdm6b in CGN differentiation

Kdm6b has known functions in multiple early developmental events, mostly in removing PcG repression to activate cell type-specific gene expression. Our study has identified expression and functions for Kdm6b in differentiated post-mitotic neurons. We have discovered that Kdm6b is robustly expressed during the development of CGNs. Its expression pattern in differentiating CGNs suggests that the H3K27 methyl mark it regulates is dynamically regulated during differentiation and maturation of CGNs. Multiple genome-wide studies have been carried out to characterize changes in chromatin modifications at lineage establishment events, when ES cells differentiate into more specialized cell types. These studies have found out that there are substantial differences in chromatin modifications between ES cells and committed progenitor cells. For example, the repressive H3K27me3 mark is removed from many neural-specific genes when ES cells differentiate into NSCs. However, we do not yet know how histone methyl marks change during the later stages of cellular differentiation, except for one study that followed H3K27 methylation on gene promoters during hippocampal granule neuron differentiation (Mohn et al., 2008). Intriguingly, this study suggests that dynamic changes in histone methylation occur throughout hippocampal granule neuron differentiation. However, this study was limited to known promoter regions, and excluded other regulatory regions like enhancers.
that could play an equally important role in determining the transcription state of a gene. In our lab, we are undertaking a much more comprehensive analysis in order to identify the regulatory regions relevant in differentiation and to characterize how chromatin structure around these regions changes during differentiation.

4.4 Epigenomics of cerebellar development

Histone modifications have become a useful way to assess chromatin state, but they are not the only changes in chromatin that can regulate genes. Other important chromatin-based gene regulatory mechanisms include the direct modification of DNA by methylation and hydroxymethylation and the remodeling of histones to change the accessibility of gene regulatory elements.

In my thesis I have focused my efforts on the study of the histone demethylationase Kdm6b. However, the CGN differentiation system I have established has great utility for asking broader questions about the changes in chromatin that occur all across the genome as CGNs differentiate. Cerebellar granule neurons (CGNs) are an ideal model system for genome-wide biochemical studies of chromatin. Large numbers of committed CGN precursors can be purified to near complete homogeneity from the postnatal mouse brain and grown in culture to discrete stages of differentiation, facilitating longitudinal analyses of chromatin over development. Thus, in addition to the work described
in detail here, over the course of my thesis research I have also participated in a genome-wide study to map chromatin regulation in differentiating CGNs.

This study takes advantage of a technique called DNase-Seq. The basic idea behind this technique is that changes in chromatin architecture allow differential access of DNA regulatory sequences to transcription factors – heterochromatic regions have less access and euchromatic regions have greater access. Regulatory elements (promoters, enhancers, silencers) that are active are generally found in open chromatin regions. Open chromatin regions are sensitive to DNAsel digestion, and open chromatin regions can be identified as DNAse hypersensitivity sites (DHS). When followed by high-throughput sequencing, this technique allows for genome-wide analysis of open chromatin regions. When combined with ChIP, we will be able to assess Kdm6b occupancy and H3K27me3 modifications at these regulatory regions during CGN differentiation. Correlations between Kdm6b occupancy and decreases in H3K27me3 levels would suggest that Kdm6b is functioning at these regulatory regions to change histone methylation. This assumption could be tested by assessing the H3K27me3 levels in Kdm6b KD CGNs, where we would expect to see the persistence of this methyl mark if Kdm6b is functioning as a demethylase.

4.3 Concluding remarks

My dissertation research has enhanced knowledge of the H3K27-specific HDM Kdm6b in several ways. My data provide the first evidence for neuronal
activity-regulated *Kdm6b* expression in post-mitotic neurons, and raise the possibility that Kdm6b may play a role in modulating programs of gene expression that contribute to hippocampal plasticity. I have identified novel functions for Kdm6b in neuronal development, survival, and differentiation. The potential Kdm6b target genes uncovered in my study include genes that encode synaptic receptors, suggesting the potential physiological functions of Kdm6b-dependent gene regulation in the developing cerebellum. Together these data identify a novel function for Kdm6b in the regulation of gene expression in differentiating cerebellar granule neurons, and raise the possibility that Kdm6b may play a role in the maturation of cerebellar neurons during development. The identification of potential target genes of Kdm6b in cerebellar granule neurons opens the door to exploring the mechanisms associated with epigenetic regulation of gene expression.
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Biography

Ranjula Wijayatunge was born in Colombo, Sri Lanka. She attended Devi Balika Vidyalaya in Colombo. She then attended Middlebury College in Vermont where she was a College Scholar and majored in Molecular Biology and Biochemistry with minors in Studio Art and Religion. Following college, she worked at the laboratory of Dr. Laurel Raftery at Massachusetts General Hospital/Harvard Medical School studying TGFbeta signaling in Drosophila. She came to Duke University through the Developmental Biology program in 2005, and joined Anne West’s lab in 2006.