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In vivo regulators of neural stem cell development and function

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Contributions

All of the work described below was designed and performed under the supervision of Dr. Daniel A. Lim

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Chapter 3 is a manuscript in preparation for submission with the following authors: Sung Jun Hong, David Wu, Eugene Gil, Dae Hwi Park, Ryan N. Delgado, Martina Malatesta, John Liu, and Daniel A. Lim. I designed, performed, and analyzed experiments. David Wu performed bioinformatics analysis for the single cell RNA-seq experiment. Daniel Lim supervised the research.

***in vivo* regulators of neural stem cell development and function**

Sung Jun Hong

Abstract

Neural stem cells in the brain give rise to both neurons and glia cells during embryonic development and help maintain tissue homeostasis in adulthood. Although transcription factors and intracellular signaling pathways that modulate NSC function in embryonic and adult brain have been heavily studied, *in vivo* functions of long noncoding RNAs (lncRNAs) and chromatin regulators in NSCs are still poorly understood. *Pnky* is a nuclear-enriched lncRNA that is transcribed divergently from the neighboring proneural transcription factor *Pou3f2*. In the embryonic cortex, I found that *Pnky* deletion increases neuronal differentiation and depletes NSCs prematurely, resulting in defects in cortical laminar structure in postnatal mice. *Pnky* expression from a bacterial artificial chromosome (BAC) transgene rescues the *in vivo* phenotypes of *Pnky*-deleted brains, supporting the idea that *Pnky* acts in trans as a key regulator of NSC function and neurogenesis in the embryonic cortex. Chromatin regulator JMJD3 is a histone demethylase implicated in development and disease of multiple organs. My studies show that *Jmjd3*-deletion in the hippocampus results in depletion of adult NSCs. During development, *Jmjd3*-deleted dentate gyrus precursors precociously differentiate into neurons, resulting in failed establishment of the hippocampal NSC niche. Single cell RNA-sequencing reveals a broad disruption of genes involved in maintaining stem cell function in *Jmjd3*-deleted NSCs. In the adult brain, loss of *Jmjd3* similarly leads to

precocious neuronal differentiation, reflecting the loss of gene expression signatures related to stem cell maintenance. These data indicate both lncRNA-*Pnky* and JMJD3 may control the rate of neurogenesis, acting like a cell-intrinsic clock for NSCs.

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Chapter 1: Introduction

During both development and adulthood, neural stem cells (NSCs) generate the diverse cell neural and glial cell types in the brain through a balance of self-renewal and differentiation. NSC populations are maintained through precisely regulated self-renewing divisions or differentiation into specific cell types. Precise regulation of NSCs is crucial for proper development, long-term tissue homeostasis, and response to tissue damage. Deficits in these processes have been associated with many human neurological diseases, which further highlights the importance of NSCs (Rubin et al., 2014; De Rubeis et al., 2014; Varela-Nallar et al., 2010). Exact spatiotemporal regulation of NSC function and output is necessary to maintain NSCs and ensure the proper differentiation into multiple neuronal cell types. Many cell-intrinsic mechanisms and extracellular signaling pathways involving transcription factors that maintain NSC identity have been discovered (Urban et al., 2014). But it is now clear that other classes of genes, including long non-coding RNAs (lncRNAs) and chromatin regulators play roles in regulating core stem cell functions (Andersen and Lim, 2018). LncRNAs are a diverse class of transcripts that may function via multiple mechanisms. Due to their intricate structures and heterogeneity, it has been difficult to study their genetic and molecular mechanisms. Similar to lncRNAs, chromatin regulators are also a diverse class of genes with multiple subgroups that can modulate gene expression in a multitude of ways. To understand how lncRNAs and chromatin regulators affect NSC function throughout development and in adulthood, *in vivo* studies that can target their genetic deletion with cell-type specificity are required.

Background

Section 1: Neural Stem cells from development to adulthood

Neural stem cells (NSCs) are a unique population of cells in the brain that can either undergo self-renewal through proliferation or differentiate into diverse cell types, including neurons and glia (astrocytes and oligodendrocytes). In the developing mouse brain, shortly after the neural tube closure at embryonic day 9.5 (E9.5), neuroepithelial (NE) cells undergo massive proliferation to expand the pool of neural stem cells (Molyneaux et al., 2007). These NE cells then transform into embryonic neural stem cells that are located in the ventricular zone (VZ) lining the ventricles. The NSCs of the embryonic brain are the radial glia (RG), so termed, because they express molecular markers of glial cells and they extend elongated radial processes away from VZ (Kriegstein and Alvarez-Buylla 2009). Embryonic NSCs can generate either excitatory projection neurons or inhibitory interneurons, depending on their location in the embryonic brain (pallium and ganglionic eminences, respectively) (Delgado et al., 2015).

The neocortex is a dorsal brain structure consisting of six layers of excitatory projection neurons. Over the course of development, excitatory neurons of the cortex are generated sequentially in an “inside-out” manner (Molyneaux et al., 2007; Rubenstein, 2011). The laminar identity of newly born neurons is correlated with the time point at which they were produced from NSCs. Transplantation studies show that as development progresses, the fate potential of NSCs to generate specific subtypes become progressively more limited (Frantz and McConnell, 1996). When earlier stage NSCs are transplanted into later stage neocortex, they are able to produce all subtypes.

However, if later stage NSCs are transplanted into earlier stage, they can only produce “later-stage neuronal subtypes” (Desai and McConnell, 2000). These data suggest that there may be a cell-intrinsic clock which controls the temporal progression of NSCs. Besides a cell-intrinsic clock, recent studies show that extracellular Wnt signaling pathway allows later stage embryonic NSCs to re-enter earlier stage states, highlighting importance of the cell-extrinsic factors for NSCs (Oberst et al., 2019). Interestingly, defects in cortical NSCs have been associated with human neurodevelopmental disorders (Lodato and Arlotta, 2015; Molyneaux et al., 2007; Rubenstein, 2011).

Most embryonic NSCs stop proliferating around the time of birth, at which point a majority of cells in the brain have been produced. In the adult brain, however, a small number of NSCs continue to proliferate and retain neurogenic potential in restricted regions. These NSCs establish adult NSC niches in the brain and generate neurons throughout life to enable circuit plasticity (Bond et al., 2015). Adult NSCs in the ventricular-subventricular zone (V-SVZ) give rise to multiple subtypes of inhibitory interneurons in the olfactory bulb (OB) (Lledo et al., 2008). Similar to embryonic NSCs, V-SVZ adult NSCs also share characteristics of glial cells, and NSCs localized to different positions rostro-caudally along the V-SVZ produce distinct neuronal subtypes (Kriegstein and Alvarez-Buylla, 2009). A retroviral barcoding experiment has shown that V-SVZ NSCs are derived from a subset of embryonic NSCs that is set aside during development until their re-activation in adulthood (Fuentealba et al., 2016).

The dentate gyrus (DG) of hippocampus is another major adult NSC niche. In the DG, NSCs are located in a subgranular zone (SGZ) where they give rise to excitatory granule neurons throughout life (Nicola et al., 2015). Similar to V-SVZ NSCs, SGZ

NSCs also have radial processes that extend from the SGZ to the granular cell layer (Kitabatake et al., 2007). SGZ NSCs arise from a subset of eNSCs located in the dorso-medial part called primary dentate neuroepithelium (DNE) (Altman and Bayer, 1990). Unlike the “set-aside” model proposed to describe the origin of V-SVZ NSCs, recent lineage tracing and fate-mapping experiments suggest a “continuous” model for the development of the SGZ NSC niche. In the “continuous” model, embryonic NSCs from DNE generate additional embryonic NSCs and granule neurons to form the dentate gyrus structure, then these embryonic NSCs acquire adult NSC properties in the SGZ during postnatal development and continue to generate DG granule neurons in the adult (Berg et al., 2020). Transcriptomic analysis shows high similarity in molecular signature between SGZ NSC precursors, postnatal NSCs, and adult NSCs further supporting the “continuous” model of their developmental origin (Berg et al., 2020).

One essential element of NSC function is the “timed” decision of whether to undergo self-renewal or differentiate into a specific cell type. Disrupting this decision, (e.g. by prematurely differentiating or excessively proliferating) can have dramatic effects on both embryonic and adult neurogenesis. If embryonic NSCs were to differentiate precociously at an earlier stage of the development, this would lead to depletion of the NSC pool and decrease the production of later stage neurons. In addition, given that embryonic NSCs are the developmental origin of adult NSCs, early depletion of embryonic NSCs would lead to failed establishment of the adult NSC niche (Fuentealba et al., 2015; Berg et al., 2020). Defects in embryonic and adult NSCs have been associated with depression, neuro-inflammation, epilepsy, and other numerous neurological disorders (Dranovsky and Hen, 2006; Parent et al., 2006; Anacker and

Hen, 2017). Depletion of adult NSCs results in cognitive decline and impaired spatial memory in mice (Burghardt et al., 2012).

The mechanism for long-term NSC function is influenced by both cell-intrinsic and -extrinsic factors, which include cell-cell contacts, activity, growth factors signaling, and gradients of signaling molecules (Urban et al., 2014). These require precise control in gene expression programs related to NSC identity and characteristics. Most regulators of *in vivo* NSC function have been centralized around signaling pathways and transcription factors (Notch, Bmp, Wnt, Fgf, and Shh) (Gaiano and Fishell 2000; Cheng et al., 2006; Okamoto et al., 2011; Imayoshi et al., 2010; Giachino et al., 2014). But it is now clear that lncRNAs and chromatin regulators can also modulate global gene expression. LncRNAs have been shown to regulate gene expression through multiple mechanisms (e.g., molecular decoy, scaffold, guide) and they can influence either neighboring genes or targets far away from their transcription sites in the genome (Rinn and Chang, 2012). Histone modifying enzymes are one type of chromatin regulators that can influence gene expression related to a specific type of modification it catalyzes upon histone proteins (Klose et al., 2006 & 2007). Although there are some hints from previous studies using *in vitro* systems, whether certain lncRNAs and/or chromatin regulators play critical, and cell-intrinsic roles for long-term NSC function is still poorly understood.

Section 2: Long non-coding RNAs (lncRNAs) in development and disease

The human genome produces tens of thousands of long noncoding RNAs (lncRNAs), transcripts with a length of greater than 200 nucleotides without apparent protein coding potential (Rinn and Chang, 2012; Andersen and Lim, 2018). These lncRNAs are found in a large diversity of species including yeast, plants, viruses, and mammals (Ma et al., 2013). LncRNAs exhibit significant cell and tissue-type specificity as compared to protein coding genes (Liu and Horlbeck et a., 2017). Thousands of lncRNAs are differentially expressed in various tissues and many of them are considered to be brain-specific (Clark et al., 2018; Quan et al., 2017). Interestingly, brain-specific lncRNAs show higher evolutionary conservation as compared to other tissues. Accumulating evidence suggests that lncRNAs can regulate fundamental biological processes, and the dysfunction of some lncRNAs has been associated with human diseases (Batista and Chang, 2013; Briggs et al., 2015). However, it remains unclear how lncRNAs influence stem cell function during development, underscoring a need to carefully dissect cell and region-specific functions of these molecules. (Liu and Horlbeck et al., 2017; Lipovich et al., 2014; Kadakkuzha et al., 2015).

lncRNAs can be classified based on their genomic location and orientation with respect to neighboring protein-coding genes (Mattick and Rinn, 2015). Some lncRNAs are transcribed antisense and overlapping with protein coding genes. Intronic lncRNAs can be either antisense or in the sense direction. Divergent lncRNAs share regulatory elements with the neighbor protein-coding gene and are transcribed in the opposite direction. Finally, intergenic lncRNAs are located further away from nearby protein coding genes and have their own regulatory elements (Andersen and Lim., 2018).

An additional layer of complexity comes from the fact that lncRNAs can regulate transcriptional regulation in *cis* – regulating neighboring genes - and/or in *trans* – modulating the function of genes at locations away from its production site (Andersen and Lim, 2018). A class of divergent lncRNAs are strongly predicted to regulate their gene neighbors, suggesting their preferential function as *cis* regulators. *Paupar* (lncRNA divergent to *Pax6*), *Six3OS* (lncRNA divergent to *Six*), and *Lnc-Brn1a* (lncRNA divergent to *Pou3f3*) are a few examples of *cis*-acting divergent lncRNAs involved in neural development (Pavlaki et al., 2018; Rapicavoli et al., 2011; Sauvageau et al., 2013). *Firre* has shown to be a *trans*-acting lncRNA involved in hematopoiesis (Lewandowski et al., 2019). Importantly, lncRNAs certainly can play multiple distinct roles both in *cis* and in *trans* (Cajigas et al., 2018; Pavlaki et al., 2018; Feng et al., 2006). The mechanisms of by which lncRNAs can influence transcriptional regulation have been investigated, and these include acting as a decoy, guide, and scaffold (Rinn and Chang, 2012). LncRNA-*PANDA* was recently discovered to act as a “decoy” lncRNA, quenching the binding site of transcription factor *NF-YA* and thereby preventing its binding to target genes that promote apoptosis (Hung et al., 2011). An example “guide” lncRNA would be *HOTAIR*, which was shown to direct chromatin modifier complex to specific regions in the genome (Rinn and Chang, 2012). *ANRIL*, on the other hand, is an example of a “scaffold” lncRNA that interacts with two chromatin modifying complexes (PRC1 and PRC2) to mediate transcriptional silencing of the *Ink4a* locus (Yap et al., 2010). While there has been some progress in understanding mechanisms of lncRNAs, the lack of good genetic tools and *in vivo* model systems have limited our understanding of their roles in development.

Some lncRNAs are involved with NSC proliferation and neuronal differentiation (e.g., *RMST, Dali, TUNA, Evf2, LncND, Six3OS*), and also in neuronal maturation and synaptogenesis (e.g., *BDNF-AS* and *Malat1*) (Quan et al., 2017). But due to the diverse nature of lncRNAs and limited scientific approaches to study them, determining exact functions have been challenging. Many studies utilize *in vitro* cell culture systems using primary cells or cell-lines from either mouse or human (Clark et al., 2012; Liu and Horlbeck et al., 2017). Tumor cell-lines are often a popular choice due to their high proliferation rate. It is clear by now that results from *in vitro* studies do not always translate into similar results *in vivo*, and such discrepancies may relate to limitations in the tools for manipulating lncRNA expression *in vivo*. Conditional knockout with traditional Cre-Lox approaches would provide cell-type specificity and spatiotemporal control over deletion of lncRNAs, but for lncRNAs that overlap with essential coding genes and/or enhancers, this approach would not work. CRISPR interference/activation approach may serve as an alternative strategy to modulate the expression of such lncRNAs (Liu and Horlbeck et al., 2017). Also, knock-down approaches using shRNA, siRNA, or antisense oligonucleotides (ASO) may also be used. But all of these approaches carry the possibility of potential off-target effects and for sh/siRNA, partial knockdown may make it difficult to identify phenotypes (Kaczmarek et al., 2017). Therefore, each lncRNA may require a different approach to investigate its function(s) in biology.

Pnky is a neural specific intergenic lncRNA neighboring *Pou3f2* (*Brn2*), an essential neural transcription factor for neocortical development and neural reprogramming (Sugitani et al., 2002; Vierbuchen et al., 2010). While *Pnky* does not

overlap with protein coding genes, it is transcribed in a divergent direction to *Pou3f2,* and *Pnky* has its own distinct regulatory elements. It can thus be classified as an intergenic lncRNA. In NSC cultures, knockdown of *Pnky* leads to increased neuronal lineage commitment and the proliferation of intermediate progenitor cells (Andersen and Lim, 2018). These results lead to an overall increase in neuronal production *in vitro*. Surprisingly, knock-down of *Pnky* in NSC *in vitro* cultures does not change the expression of *Pou3f2* or other neighboring genes within a 5MB range, suggesting that *Pnky* does not regulate gene expression in *cis.* Fluorescent *in situ* hybridization experiments show that several (>2) *Pnky* transcripts are detected in the nucleus, further suggesting function in *trans* (Ramos and Andersen et al., 2015). The exact function of *Pnky in vivo*, however, is still poorly understood. Since *Pnky* does not overlap with any neighboring coding genes and has its own promoter, it is an ideal candidate for traditional genetic deletion approaches to study cell-intrinsic functions of *Pnky* in NSCs *in vivo*.

Section 3: Chromatin regulators influence stem cell function

Alterations of chromatin landscape and gene expression changes are closely associated with each other. Histone modifiers are one type of chromatin regulators that modulate gene expression by attaching chemical modifications to histones. The N-terminal tail of each core histone protein is subject to various types of post-translational modifications (PTMs). Acetylation, methylation, phosphorylation, and ubiquitination are the most common types of histone modifications (Wang et al., 2013). These site-specific PTMs influence various steps involved in RNA transcription such as activation,

repression, elongation, and termination to modulate gene expression (Mellor, 2006) and are each catalyzed by specific histone-modifying enzymes. Histone modifiers have been associated with fundamental processes of neural development, and their dysfunction has been implicated in human neurological disorders (Mirabella et al., 2016).

Jumonji domain-containing protein D3 (JMJD3), also called lysine(K)-specific demethylase (*Kdm6b*), was discovered as a histone 3 lysine 27 (H3K27) specific demethylase (Hong et al., 2007; Swigut et al., 2007). It is part of a protein family that contains a *JmjC* domain as its catalytic domain required for its demethylase activity (Klose et al., 2006). Promoter regions of transcriptionally repressed genes are enriched with H3K27me3, a chromatin mark associated with transcriptional repression. Therefore, through JMJD3's demethylase activity, genes appear to be de-repressed, allowing for the activation of gene expression programs.

JMJD3 has been implicated in various aspects of stem cell regulation from development to adulthood. In embryonic stem cells (ESCs), *Jmjd3* is involved in the formation of the three germ layers (endoderm, mesoderm, and ectoderm) (Burchfield et al., 2015). Importantly, *Jmjd3*-deletion does not seem to affect self-renewal (Mansour et al., 2012; Ohtani et al., 2013). Previous studies have shown that JMJD3 interacts with germ-layer specific transcription factors to activate lineage-specific genes in embryonic stem cells through interaction with various signaling pathways (WNT, TGF-β, BMP, and SMAD) (Burchfield et al., 2015). In adult V-SVZ NSCs, JMJD3 is required to activate key neurogenic genes via interactions at not only promoter but also enhancer regions. Through activation of those essential neural genes, NSCs are able to give rise to olfactory bulb neurons (Park et al., 2014). Based on published studies in multiple model

systems, it seems that JMJD3 is expressed across multiple cell and tissue types. JMJD3 can regulate differentiation, stem cell proliferation, or lineage specification, cellular reprogramming, and tissue repair (Martinelli et al., 2011; Zhao et al., 2013; Yasui et al., 2011). These functions of JMJD3 and other chromatin regulators depend on cell and tissue-type specificity, suggesting that the exact function and molecular mechanism of JMJD3 may be context-dependent. But whether JMJD3 has a cell-intrinsic function for the establishment and long-term maintenance of NSCs in the hippocampus is not known. In addition, most known functions of JMJD3 have been identified using *in vitro* cell cultures (Burchfield et al., 2015). Its exact role *in vivo* would further enhance our understandings about chromatin biology and NSC functions.

While most embryonic NSCs produce neurons in the developing brain, a subset of them transition to establish adult NSC pool in restricted regions where they last for the life of the animal (Bond et al., 2015). Mechanisms of regulating embryonic NSC function, transition from embryonic to adult NSCs, and maintenance of NSCs for life-long neurogenesis are poorly understood. There have been some hints from studies of major signaling pathways. Notch has been shown to be essential for NSC maintenance. Loss of essential Notch components, RBPJk or Jagged1, in embryonic NSCs leads to proliferation defects as well as precocious neuronal differentiation causing embryonic NSC depletion (Imayoshi et al., 2010). The function of bone morphogenic proteins (BMPs) on embryonic NSCs is diverse, and depending on a specific BMP receptor (BMPR-1a or BMPR-1b), BMPs regulates either proliferation or neuronal differentiation (Panchision et al., 2010). In adult NSC, WNT signaling promotes neuronal differentiation, where knockdown of *Wnt5a* leads to reduced number of newly born

neurons in adult hippocampus (Arredondo et al., 2019). Another component of WNT signaling, SFRP3, has been shown to regulate adult NSC maintenance, where loss of SFRP3 leads to depletion of quiescent NSC pool (Jang et al., 2013). Essential neurogenic genes (Ngn2, NeuroD1, and Prox1) are also induced by WNT signaling pathways (Kuwabara et al., 2009). In this dissertation, I have studied lncRNA *Pnky* and the chromatin regulator JMJD3 play critical roles in the maintenance of NSCs during development and in the adult mouse brain.

Chapter 2: The long noncoding RNA *Pnky* is a trans-acting regulator of neural stem cells *in vivo*

Summary

Despite the recent identification of thousands of lncRNAs, few have been definitively shown to regulate specific cellular events *in vivo* especially with genetic tools that can distinguish between *cis* vs *trans* mechanisms. *Pnky* is a nuclear-enriched lncRNA that is transcribed divergently from the neighboring proneural transcription factor *Pou3f2*. Here, I show that *Pnky* from the embryonic cortex regulates the rate of the neuronal production, where *Pnky* deletion leads to precocious neuronal differentiation of NSCs and exhausts NSC prematurely. This early depletion of NSCs during embryonic development alters postnatal cortical lamination. Surprisingly loss of *Pnky* does not influence the expression level of neighboring gene, *Pou3f2*. *Pnky* expression from a bacterial artificial chromosome (BAC) transgene rescues the *in vivo* phenotypes of *Pnky*-deleted NSCs. Thus, I find that *Pnky* regulates NSC function in the embryonic cortex by acting in *trans*.

Introduction

Long non-coding RNAs (lncRNAs) are transcripts greater than 200 nucleotides without protein coding potential that are produced by the mammalian genome. They are differentially expressed in various tissue and disease states, and many have been suggested to play essential roles in fundamental cellular processes (Rinn and Chang 2012; Andersen et al., 2018). Though the level of expression of lncRNAs is generally lower than that of protein-coding genes, lncRNAs are highly cell-type specific (Liu and Horlbeck et al., 2017). Therefore, deleting lncRNAs in a cell type- or tissue- specific manner is crucial to better understand their roles. Even for lncRNAs for which functions have been explored, the exact mechanism by which those lncRNAs regulate gene expression is still unclear (Kopp and Mendell, 2018). LncRNAs can functions in *cis* – regulating neighboring genes - and/or in *trans* – modulating the function of genes at locations away from its production site (Andersen and Lim, 2018). Understanding how lncRNAs regulate biology (*cis* vs *trans*) in vivo system is crucial for the lncRNA field.

Pnky is a nuclear-enriched lncRNA expressed in NSCs in both the human and mouse brain. This 825nt lncRNA is an example of an intergenic lncRNA, which gets transcribed divergently in the opposite direction from a neighboring transcription factor, *Pou3f2* (Ramos and Andersen et al., 2015). Previous studies using short-hairpin RNA (shRNA)-mediated *Pnky* knockdown (KD) show that loss of *Pnky* leads to increased neuronal differentiation *in vitro* NSC cultures. However, owing to potential off-target effects and low shRNA KD efficiency, it has been difficult to determine the exact mechanism of action of *Pnky* using this approach. This observation belies a need for an alternate strategy to modulate *Pnky* expression

In this chapter, I describe a series of experiments utilizing multiple genetic approaches to investigate the *in vivo* function and mechanism of *Pnky* in NSCs during development. I generated mice carrying a conditional allele of *Pnky* and used *Emx1-Cre* mediated recombination to delete *Pnky* specifically in the embryonic cortex. Deleting *Pnky* in cortical NSCs and their progeny phenocopied previously reported results from studies using shRNA construct to knockdown *Pnky* (Ramos and Andersen et al., 2015). Importantly, the disruption of *Pnky* did not influence the expression of the neighboring transcription factor, *Pou3f2*, raising the hypothesis that *Pnky* may act in *trans* (Ramos and Andersen et al., 2015; Andersen and Hong et al., 2019). To directly test this hypothesis, I generated another transgenic mouse carrying an integrated bacterial artificial chromosome (BAC) construct that expresses *Pnky* at physiological levels was generated (Andersen and Hong et al., 2019). Expression of *Pnky* from the BAC was sufficient to rescue *in vivo* phenotypes observed in *Pnky*-deleted mice. Collectively, these data from multiple genetic studies show that *Pnky* regulates NSC function in *trans* to regulate neural development.

Results

Generation of a conditional *Pnky* deletion allele

In order to obtain tissue- and cell-type specific control over *Pnky* deletion *in vivo*, I used a Cre-loxP approach. In the design of this genetic approach, I remained agnostic about the specific mechanism(s) by which *Pnky* might function. Therefore, a mouse was produced carrying a conditional “floxed” Pnky allele (*Pnky*^{F}), in which the entire *Pnky* gene including its transcriptional start site (TSS) was flanked by loxP sites (**Figure**

2.1A). Interestingly, conditional deletion of the entire *Pnky* locus in *in vitro* NSC cultures produced similar phenotypes as those observed using an shRNA-mediated *Pnky* KD, including 3~4 fold increase in neuron production (Andersen and Hong et al., 2019).

Pnky deletion leads to increase neuronal differentiation *in vivo*

The neocortex is a highly organized dorsal brain structure comprised of six layers of excitatory projection neurons. The proper development of these layers is critical to cognitive function and defective development has been associated with human neurodevelopmental disorders (Lodato and Arlotta, 2015; Molyneaux et al., 2007; Rubenstein, 2011). Embryonic NSCs in the neocortex produce multiple subtypes of projection neurons. Aside from projection neurons, the neocortex also contains inhibitory interneurons generated from NSCs in ventral regions (subpallium) that migrate into the cortex during development. Throughout embryonic development, the relative abundance of one neuronal type can influence the development of another. For example, changes in the production of cortical interneurons can influence the generation of projection neurons, and vice versa (Lodato et al., 2011). *Pnky* transcripts are detected in both the pallium and subpallium of the developing brain, suggesting roles for *Pnky* in the generation of both excitatory and inhibitory neurons. (Ramos and Andersen et al., 2015). I focused my analysis on pallial NSCs, in order to investigate the role of *Pnky* in stem cell maintenance of the projection neuron lineage.

I targeted *Pnky*-deletion to pallial NSCs by crossing mice expressing Cre under the control of the “empty spiracles homeobox 1” promoter (*Emx1-Cre*) with mice carrying conditional alleles of *Pnky* (*Pnky*$^{F/F}$) (Gorski et al., 2002). *Emx1* drives Cre expression in the pallium beginning at ~ embryonic day (E) 9.5 (Briata et al., 1996;

Gorski et al., 2002; Simeone et al., 1992). *In situ* hybridization (ISH) detected *Pnky* expression in the pallial VZ at E10.0 in control animals, whereas its expression was ablated in the pallium of *Emx1-Cre; Pnky*$^{F/F}$ mice, and maintained in the subpallium (Andersen and Hong et al., 2019).

The laminar structure of neocortex develops in an “inside-out” manner: NSCs first give rise to deep layer (DL) projection neurons (Layers 5 and 6), while later-born neurons migrate past them to form the upper layers (Layers 2-4) (Lodato and Arlotta, 2015). In mice, the birth of DL neurons peaks at approximately E13.5. In *Emx1-Cre;Pnky*$^{F/F}$ cortices at E13.5, I observed increased numbers of CTIP2+ subcerebral neurons in the cortical plate (CP) (+24.3%) compared to littermate controls (**Figures 2.1B-C**). This increase in neuronal production was accompanied by a decrease in proliferating VZ, as determined by quantification of cells expressing the mitotic indicator phosphorylated histone H3 (pH3) (-17.3%) (**Figures 2.1D-E**) and the radial glial marker phosphorylated Vimentin (pVIM) (-11.8%) (**Figures 2.1F-G**). Thus, consistent with prior results from shRNA-mediated *Pnky* KD (Ramos and Andersen et al., 2015), conditional deletion of *Pnky (Pnky*-cKO) leads to an increase in neuronal differentiation and an accompanying but decrease in the number of proliferative NSCs. These findings suggest that *Pnky* is necessary to maintain proliferative NSCs.

With the increase in early-born CTIP2+ neurons at E13.5 (**Figures 2.1B-C**), I then followed up at a postnatal time point when the laminar structure of the cortex has been set up, allowing to evaluate the longer term effects of *Pnky* deletion on the abundance of neurons in specific layers. By P14, the laminar structure of neocortex is fully visible. Consistently with the early increase in the generation of CTIP2+ neurons at

E13.5, I observed an increase (+8.1%) in the number of CTIP2+ neurons in layer (L) 6 in *Emx1-Cre; Pnky*$^{F/F}$ mice compared to controls (**Figures 2.2A-B**). As I also observed decreased numbers of proliferative NSCs at E13.5, I reasoned that UL neurogenesis may be impaired in our mutants, since most UL neurons are generate later, ~E15.5. Consistent with this expectation, the P14 cortex of *Emx1*-Cre;*Pnky*$^{F/F}$ mice contained fewer (-14.4%) CUX1+ UL neurons compared to littermate controls. (**Figures 2.2C-D**).

Generation of BAC^{Pnky} mice

Unlike with divergent lncRNAs, whose deletion influences the expression level of neighboring genes, *Pnky* deletion did not affect the expression level of the neighboring *Pou3f2* transcription factor. The lack of evidence for *Pnky* regulating *Pou3f2* or other genes in *cis*, led us to hypothesize that *Pnky* may regulate gene expression in *trans.* I thus acquired a bacterial artificial chromosome (BAC) containing ~170kb of the genomic sequence surrounding *Pnky* but lacking the coding sequence of Pou3f2 (only other complete gene in this construct) (**Figure 2.3A**). I then generated transgenic mice (BAC^{Pnky}) that express *Pnky* from a modified version of this BAC construct (Andersen and Hong et al., 2019).

***Pnky* functions in *trans* to regulate neural stem cell function**

To investigate whether BAC^{Pnky} can rescue the phenotype of *Pnky*-deletion *in vivo*, I generated mice with germline *Pnky*-deletion (*Pnky*$^{\Delta/\Delta}$) and crossed it to the BAC^{Pnky} line to produce *Pnky*$^{\Delta/\Delta}$; BAC^{Pnky} mice (**Figure 2.3B**). Unlike *Pnky*$^{\Delta/\Delta}$ mice, where there were no detectable *Pnky* transcripts, *Pnky*$^{\Delta/\Delta}$;BAC^{Pnky} mice exhibited a similar level of *Pnky* expression as *Pnky*$^{+/+}$ animals at E13.5 (**Figure 2.3C**). The *Pnky*$^{\Delta/\Delta}$ cortex at E13.5 had decreased (-16.6%) number of CTIP2+ neurons in the CP

compared to littermate controls (**Figures 2.3D-E**). This decrease may be due to earlier loss of *Pnky* expediting depletion of NSCs in *Pnky*$^{\Delta/\Delta}$ mice compared to *Emx1*-Cre;*Pnky*$^{F/F}$. Consistent with this idea, I observed a decrease in pH3+ NSCs in the VZ of *Pnky*$^{\Delta/\Delta}$ brains and reduced overall thickness of the neocortex (**Figures 2.3 G-H**). Surprisingly, the presence of BAC*Pnky* transgene was sufficient to reverse the phenotype observed in *Pnky*$^{\Delta/\Delta}$ mice (**Figures 2.3D, F, G, I**). Thus, these data suggest that the expression of *Pnky* in *trans* is sufficient to rescue the phenotype resulting from the loss of *Pnky in vivo*.

Postnatal laminar defect in *Pnky*Δ/Δ is rescued by BAC^{Pnky} transgene

To determine whether the decreased in number of CTIP2+ neurons in E13.5 *Pnky*$^{\Delta/\Delta}$ brain leads to persistent defects in laminar structure, I assessed P14 *Pnky*$^{\Delta/\Delta}$ brains compared to littermate controls. CTIP2+ labels both L5 subcerebral and L6 corticospinal neurons. I found a decrease in both L6 CTIP2+ (-11.7%) and L5 CTIP2+ (-12.5%) neurons; however, unlike L5, decrease of L6 did not reach statistical significance (p = 0.056) (**Figures 2.3J-J'**). In addition, P14 *Pnky*$^{\Delta/\Delta}$ exhibited a decrease in the number of CUX1+ UL neurons (-9.1%) (**Figure 2.3K**). Thus, *Pnky*-deletion leads to an overall reduction in multiple subtypes of projection neurons in the postnatal cortex. Interestingly, adult *Pnky*$^{\Delta/\Delta}$ mice (~3 months old) exhibited an impairment in acoustic startle threshold, pre-pulse inhibition, and cued fear conditioning (**Table 2.1**) suggesting its potential role in cognition. Astonishingly, even at P14, the presence of BAC^{Pnky} transgene was sufficient to rescue the phenotype resulting from *Pnky*-deletion (**Figures 2.3L-M**).

Discussion

Despite extensive effort to study the function of lncRNAs, there are relatively few genetic in vivo studies of these RNA transcripts. By using a combination of mouse genetics (conditional allele of *Pnky* & BAC^{Pnky}), I show that *Pnky* functions in *trans* to regulate eNSCs during neocortical development. Surprisingly, there was no evidence to suggest that *Pnky*-deletion influences the expression of the neighboring *Pou3f2* gene, suggesting that it does not function in *cis* (Andersen and Hong et al., 2019). Moreover, *Pnky* expression from a BAC transgene was sufficient to rescue the phenotypes observed in *Pnky*$^{\Delta/\Delta}$ mice further supporting that it functions in *trans*.

The sequential production of excitatory projection neurons during development is essential for the formation of the proper laminar structure of the neocortex. The neocortical development is regulated through the temporal progression of NSCs generating multiple neuronal subtypes depends on the timing of the neurogenesis (Frantz and McConnell, 1996). When *Pnky*-deletion was targeted to neocortical NSCs, the number of deep layer neurons in layer 6 was increased while the number of upper layer neurons was decreased. These results show that *Pnky*-deletion results in early neuronal differentiation (deep layer neurons) *in vivo* at the expense of NSCs maintenance which matches the *in vitro* phenotype previously observed with shRNA-mediated knockdown of *Pnky* in NSC cultures (Ramos and Andersen et al., 2015).

This increase in neurogenesis observed with *Pnky*-deletion is distinct compared to other known lncRNAs that have been shown to regulate neural development. For example, *RMST* is a lncRNA that interacts with SOX2 to co-regulate essential downstream neuronal genes (*Dlx1*, *Hey2*, and SP8). This lncRNA gets upregulated

during the neuronal differentiation of human embryonic stem cells. Knockdown of *RMST* in NSC culture leads to decrease in neuronal differentiation (Ng et al., 2013). Similar to *RMST*, *TUNA* is another lncRNA that regulates the neuronal differentiation by interacting with multiple RNA-binding proteins. Knockdown of *TUNA* decreases neuronal differentiation in NSC cultures (Lin et al., 2014). Knockdown of other lncRNAs such as *Six3OS* and *Dlx1as* also lead to reduction in neurogenesis. Interestingly, recently published results show that *LncND* interacts with components of Notch signaling where knockdown of *LncND* actually leads to an increase in neuronal differentiation, similar to the phenotype *Pnky*-deletion (Rani et al., 2016). Notably, most of these results are based on *in vitro* cell cultures; therefore, further *in vivo* studies are required to better understand their potential roles in neural development.

Interestingly, unlike phenotypes observed from *Emx1-Cre* mediated conditional deletion of *Pnky*, constitutive deletion leads to reduction in both deep layer and upper layer neurons in the neocortex of $Pnky^{\Delta/\Delta}$ brain. This result could be due to constitutive deletion of *Pnky* causing a depletion of NSCs (or even neuroepithelial cells) at much earlier stages of brain development. Further *in vivo* analysis at earlier embryonic time points might clarify this question. Similar to *Pnky*, *Lnc-Brn1b* is a lncRNA located ~10kb downstream of *Pou3f3* (paralog of the Pnky neighbor *Pou3f2*) (Sauvageau et al., 2013). Constitutive deletion of *lnc-Brn1b* leads to increase in deep layer neurons and decrease in upper layer neurons in the postnatal cortex, which is different from *Pnky*-specific phenotypes (Sauvageau et al., 2013). In addition, while *Pnky* does not influence the expression of *Pou3f2*, deletion of *lnc-Brn1b* leads to reduced expression level of *Pou3f3*. Divergent lncRNAs are strongly predicted to regulate its neighboring genes.

Lnc-Brn1a is a lncRNA that is divergent to *Pou3f3* and shRNA-mediated knockdown of *lnc-Brn1a* leads to reduced expression level of *Pou3f3*. *Paupar* (lncRNA divergent to *Pax6*) and *Six3OS* (lncRNA divergent to *Six*) are just other examples of divergent lncRNAs regulating neighboring coding gene neighbor, highlighting the uniqueness of *Pnky* (Pavlaki et al., 2018; Rapicavoli et al., 2011).

Results from both conditional and constitutive deletion of *Pnky* suggest that *Pnky* is required for the maintenance of NSCs in the embryonic brain. Deletion of *Pnky* results in precocious neuronal differentiation at the expense of NSCs. Surprisingly, *Pnky* is also required for long-term maintenance of adult V-SVZ NSC. Adult NSCs in the V-SVZ give rise to transit-amplifying cells and neuroblasts that migrate to the olfactory bulb where they differentiate into inhibitory interneurons.

Both *Nestin-Cre* mediated conditional deletion and constitutive deletion of *Pnky* in V-SVZ NSCs result in formation of the neuroblast nodules along the walls of the lateral ventricles (Andersen R, UCSF dissertation). BrdU labeling experiments show that *Pnky*-deletion leads to increase in neuronal differentiation suggesting increased neuronal production may contribute to the formation of neuroblast nodules in *Pnky*-deleted brains. This increase in neuronal production results in stark reduction of adult V-NSCs consistent with phenotypes observed in embryonic brain.

Based on those results, I propose that *Pnky* functions in similar ways in embryonic NSCs and adult NSCs. Moving forward, determining *Pnky*'s binding partners for its cell-intrinsic NSC function may provide critical insights into how this lncRNA functions at the molecular level to regulate long-term NSC function. Based on the

similarity between embryonic NSC phenotypes and adult NSC phenotypes, I suggest that such *Pnky*-interacting complex(es) are the same in the embryonic and adult brain.

Figures and Tables

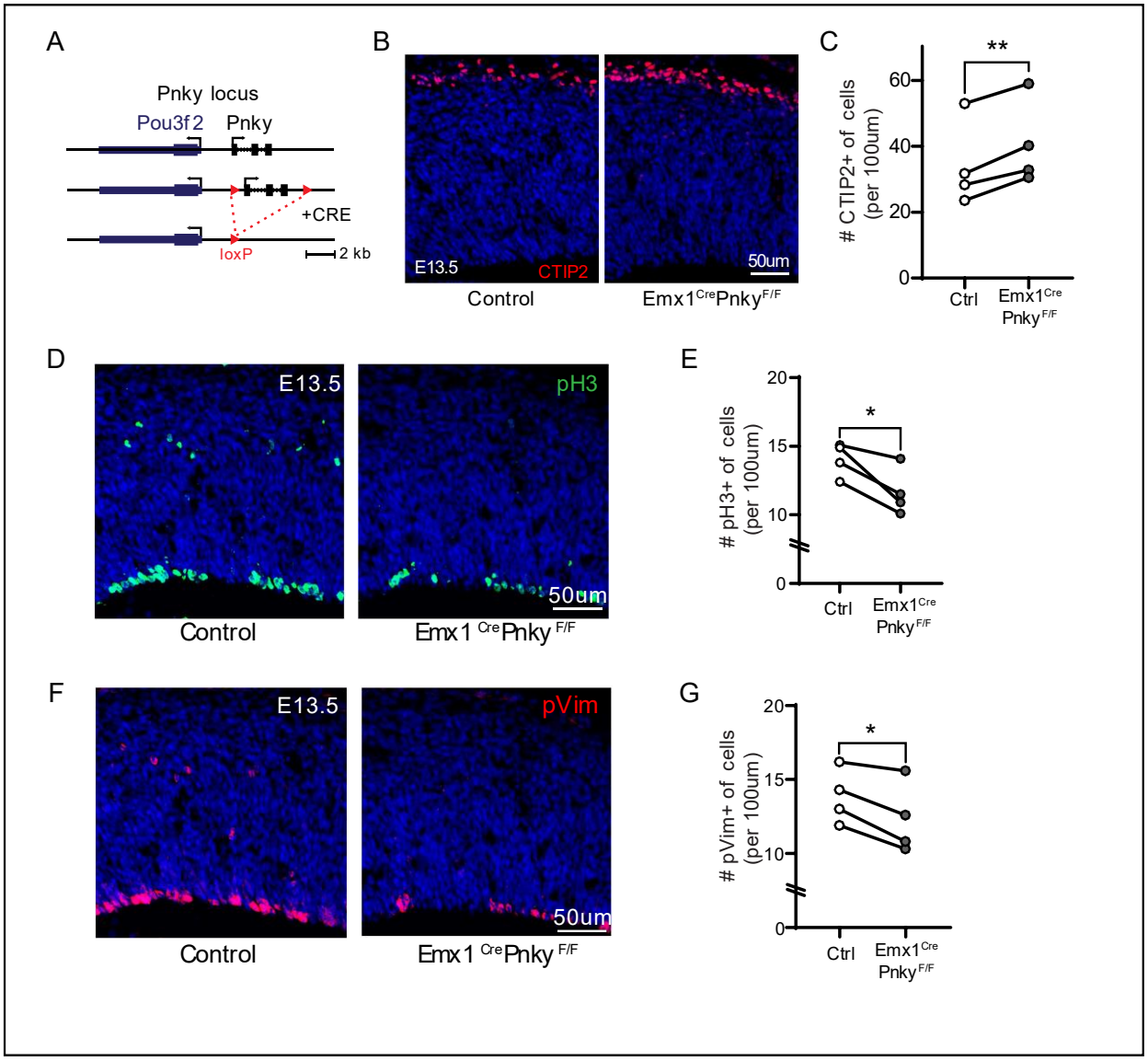

Figure 2.1 Pnky regulates the embryonic cortical neurogenesis in vivo
* = p < 0.05, ** = p < 0.01, two-tailed paired t test.
A, Schematic of Pnky locus and loxP site insertions. **B,** CTIP2 IHC with DAPI (blue). **C,** Quantification of (B). **D,** pH3 IHC with DAPI (blue). **E,** Quantification of (D). **F,** pVim IHC with DAPI (blue). **G,** Quantification of (F).

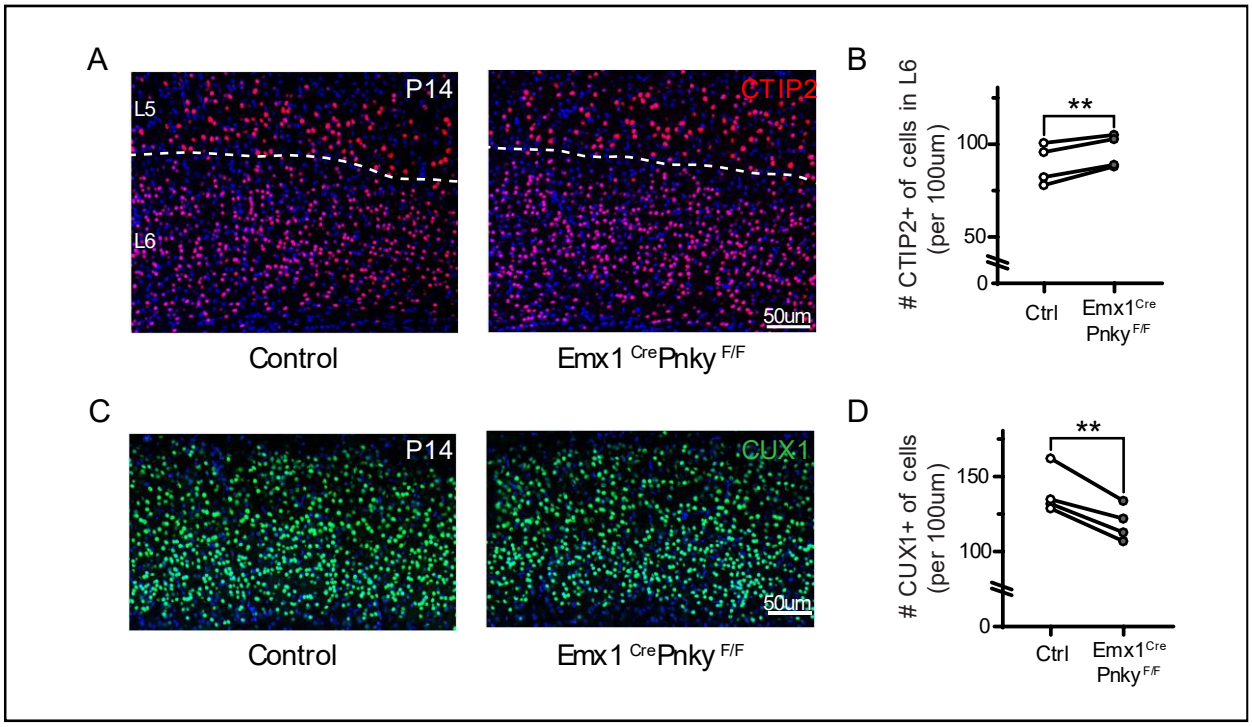

Figure 2.2 Pnky regulates the postnatal cortical neurogenesis in vivo
* = p < 0.05, ** = p < 0.01, two-tailed paired t test.
A, CTIP2 IHC with DAPI nuclear stain (blue). **B,** Quantification of CTIP2+ L6 neurons in Emx1Cre;*Pnky*$^{F/F}$ compared to littermate control, two-tailed paired t test. C, CUX1 IHC with DAPI nuclear stain (blue). **D,** Quantification of (C), two-tailed paired t test.

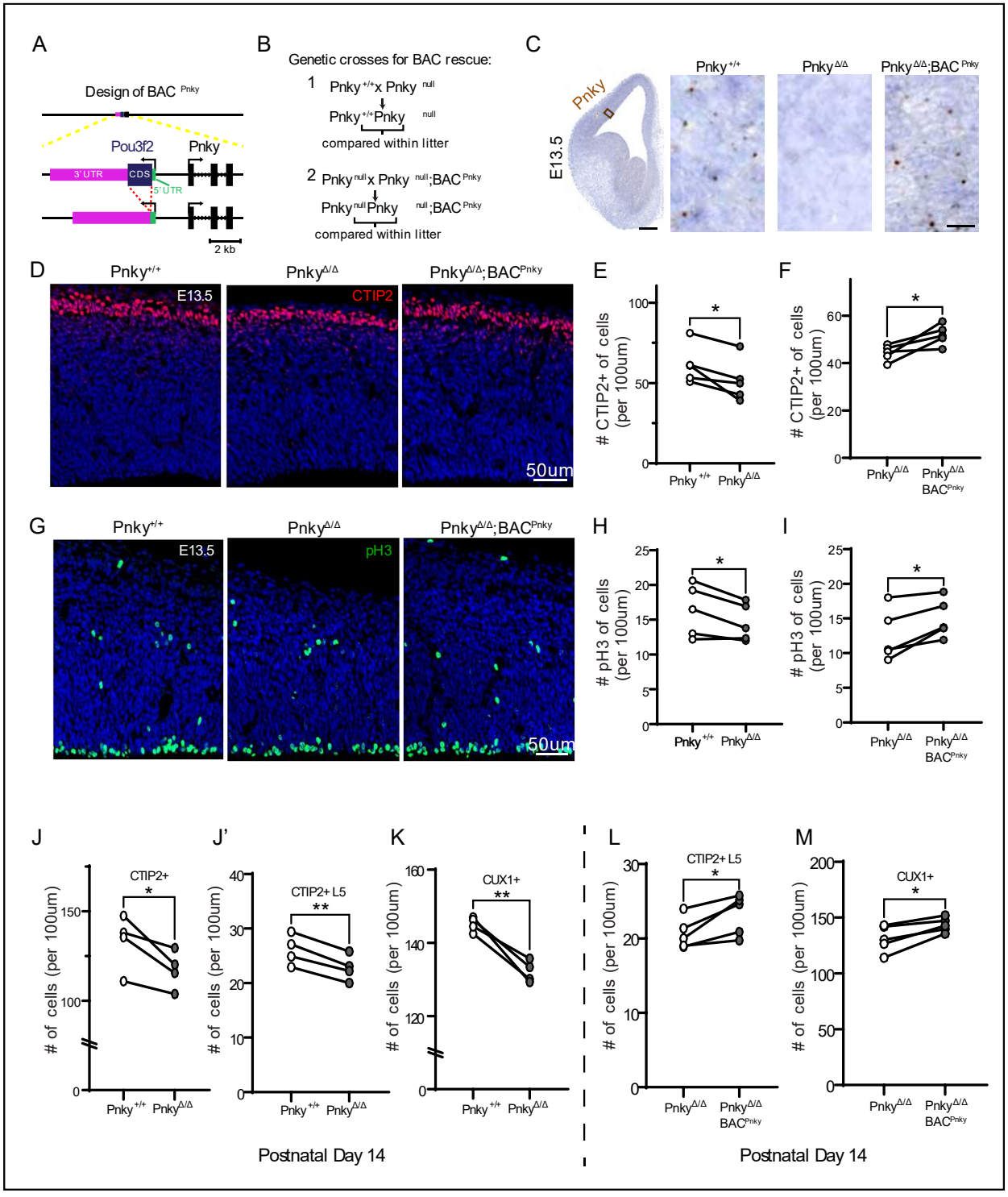

Figure 2.3 BAC transgenic expression rescues loss of the endogenous gene.
* = $p < 0.05$, ** = $p < 0.01$, two-tailed paired t test.
A, Schematic of BAC*Pnky* transgene. **B,** Diagram of genetic crosses for BAC rescue experiments. **C,** ISH of Pnky (brown puncta) with hematoxylin counterstain (blue). Representative section with red box indicating approximate region of pallium enlarged

on the right. Scale bars = 250um and 25um (inset). **D,** CTIP2 IHC with DAPI (blue). **E** Quantification of (D) comparing *Pnky*$^{\Delta/\Delta}$ to littermate control, two-tailed paired t test. **F,** Quantification of (D) comparing *Pnky*$^{\Delta/\Delta}$;BAC^{Pnky} to *Pnky*$^{\Delta/\Delta}$, two-tailed paired t test. **G,** pH3 IHC with DAPI (blue). **H**, Quantification of (G) comparing *Pnky*$^{\Delta/\Delta}$ to littermate control, two-tailed paired t test. **I,** Quantification of (G) comparing *Pnky*$^{\Delta/\Delta}$;BAC^{Pnky} to *Pnky*$^{\Delta/\Delta}$, two-tailed paired t test. **J** and **J'**, Quantification of CTIP2+ neurons in *Pnky*$^{\Delta/\Delta}$ compared to littermate control. Total CTIP2+ and L5 specific CTIP2+, respectively. **K,** Quantification of CUX1+ neurons in *Pnky*$^{\Delta/\Delta}$ compared to littermate control, two-tailed paired t test. **L,** Quantification of CTIP2+ neurons in L5 comparing *Pnky*$^{\Delta/\Delta}$;BAC^{Pnky} to *Pnky*$^{\Delta/\Delta}$, two-tailed paired t test. **M,** Quantification of CUX1+ neurons comparing *Pnky*$^{\Delta/\Delta}$;BAC^{Pnky} to *Pnky*$^{\Delta/\Delta}$, two-tailed paired t test.

Table 2.1: Summary of behavior test results between *Pnky*$^{\Delta/\Delta}$ and WT control

Behavioral Assessments	Pnky lncRNA Females (WT, n=11 & *Pnky*$^{\Delta/\Delta}$, n=12)	Pnky lncRNA Males (WT, n=11 & *Pnky*$^{\Delta/\Delta}$, n=14)
Elevated Plus maze		
-%time Open Arms	No change	No change
-Open arm/total distance	No change	No change
-Activity measures	No change	No change
Open Field activity		
-Ambulatory movement	*Trend for Increase*	No change
-Fine movement	**Decreased**	No change
-Rearing	No change	No change
Rotarod		
-Fixed speed (16 RPM)	No change	No change
-Accelerating speed (4-40 RPM)	No change	No change
Nesting		
-Nesting score	No change	No change
-Nesting score x time	No change	No change
2-Trial Social Approach		
Habituation – Trial 1 *-Interaction time* *-Interaction time/bout* *-Chamber side time*	 No change No change No change	 No change No change No change
Social Approach – Trial 2 *-Interaction time* *-Interaction time/bout* *-Chamber side time*	 No change No change No change	 No change No change No change
Object-Context Congruence		
Training Trials 1 & 2 *-Interaction bouts* *-Interaction time* *-Interaction % time*	 No change No change No change	 No change No change No change
Congruence Test – Trial 3 *-Interaction bouts* *-Interaction time* *-Interaction % time*	 No change No change No change	 No change No change No change
Acoustic Startle Threshold		
-Startle threshold	**Increased Startle**	No change
-Startle 70 vs 80 dB	No change	No change

Table 2.1: Summary of behavior test results between *Pnky*$^{\Delta/\Delta}$ and WT control (continued)

	Pnky lncRNA Females **(WT, n=11 & *Pnky*$^{\Delta/\Delta}$, n=12)**	**Pnky lncRNA Males** **(WT, n=11 & *Pnky*$^{\Delta/\Delta}$, n=14)**
Pre-pulse Inhibition		
-pure startle	**Increased Startle**	No change
-% inhibition	**Decreased % Inhibition @4dB pre-pulse stimuli**	No change
Cued Fear Conditioning		
Fear conditioning *-baseline* *-cued* *-cue ITI*	 No change No change No change	 No change No change No change
-Motion index	No change	No change
-Fear context recall	No change	No change
Cued fear recall *-baseline* *-cued* *-cue ITI*	 No change No change No change	 **Decreased freezing** **Decreased freezing** **Decreased freezing**
Hot Plate		
-Hind paw withdrawal	No change	No change
Body Weight	**Decreased**	No change

Experimental Procedures

Mus musculus

All mice were group-housed and maintained in the University of California, San Francisco Laboratory Animal Resource Center under protocols approved by the Institutional Animal Care and Use Committee. All relevant ethical regulations were followed. Mice of both sexes were used for all experiments, and were analyzed at multiple ages between E13.5 and P14 as described. For *Emx1*Cre experiments, control samples were *Emx1*-Cre;*Pnky*$^{+/+}$ or any combination of *Pnky* alleles in the absence of *Emx1*Cre. Details regarding mouse strains are as follows

UBC-Cre-ERT2: Tg(UBC-cre/ERT2)1Ejb, described in (Ruzankina et al., 2007).

*Emx1*Cre: Emx1tm1(cre)Krj, described in (Gorski et al., 2002).

Ai14: Gt(ROSA)26Sortm14(CAG-tdTomato)Hze, described in (Madisen et al., 2010).

E2a-Cre: Tg(EIIa-cre)C5379Lmgd, described in (Lakso et al., 1996).

Generation of the *Pnky* conditional (*Pnky*^{F}) mouse line

A conditional allele of *Pnky* was created by inGenious Targeting Laboratory through homologous recombination in C57BL/6 x 129/SvEv hybrid embryonic stem (ES) cells. The targeting construct contained a Neomycin resistance cassette to enable drug selection of recombined cells. Targeted ES cells were microinjected into C57BL/6 blastocysts. Resulting chimeras with a high percentage agouti coat color were mated to C57BL/6 FLP mice to remove the Neomycin resistance cassette. The resulting *PnkyF* allele has the entire *Pnky* gene flanked by loxP sites, with one loxP site 727 bp upstream of the TSS and the other 938 bp downstream of the transcriptional end site (TES) (Andersen and Hong et al., 2019).

Generation of the *Pnky*$^{\Delta/\Delta}$ mouse line

Pnky$^{\Delta/\Delta}$ mice was obtained through crossing mouse carrying conditional alleles of Pnky (Pnky$^{F/F}$) with the germline-expressed E2a-Cre mouse line. Progeny with germline deletion of *Pnky* were then mated with wild type mice to breed out the E2a-Cre transgene.

Generation of the BAC*Pnky* mouse line

BAC clone RP23-451I6 was obtained from the BACPAC Resources Center and modified to remove the coding sequence (CDS) from *Pou3f2*, leaving the UTRs intact (Andersen and Hong et al., 2019). Modifications were made as described in (Warming et al., 2005), and using the detailed protocols found at https://ncifrederick.cancer.gov/research/brb/protocol.aspx. I primarily used "Recombineering Protocol #3" and referred to "Recombineering Protocol #1" for additional details. The modified BAC was microinjected into C57BL/6 zygotes by the Transgenic Gene Targeting Core (Gladstone Institutes, UCSF).

Mouse Behavior Test

Behavior test of *Pnky*$^{\Delta/\Delta}$ and WT controls were performed and analyzed by the behavioral core at Gladstone, UCSF.

Polymerase chain reaction (PCR)-based genotyping of *Pnky* and BAC*Pnky*

DNA from clipped tail samples was prepared by boiling in 100μL of lysis buffer (0.2mM EDTA, 25mM NaOH) at 100^{o}C for 1 hr, followed by quenching in 100μL of neutralization buffer (40mM Tris HCl pH 7.5). Genotyping was performed using GoTaq (Promega, M3001) and the following reaction components, for a final volume of 12μL per reaction: 7.76μL of H2O, 2.4μL of 5x buffer, 0.36μL of 50mM MgCl2, 0.24μL of

10mM DNTPs, 0.12µL of primer mix (with each primer at 10µM), 0.12µL of GoTaq, 1µL of sample DNA. Reactions were incubated on a thermocycler as follows: 94°C for 2 min; 35 cycles of 94°C for 30 seconds (s), 60°C for 30 s, and 72°C for 1 min; 72°C for 5 min; 4°C hold. Reaction products were separated on a 2% agarose gel with ethidium bromide. The following primers were used (all listed 5' to 3'):

Primers for *Pnky*^{F}, *Pnky*$^{+}$, and *Pnky*$^{\Delta}$ alleles:

Pnky GT F: TAAGCTCAAACTCCGGTCCCGGGA

Pnky GT R1: TCAGGGACAAAGAACCAAAACGAGC

Pnky GT R2: AATGCTCCCTCTGAGCCTCAATT

Reaction products: 120bp (*Pnky*null), 221bp (*Pnky*+), and 348 (*Pnky*F). Since BAC*Pnky* contains unaltered *Pnky*, this will produce the 221bp product, even in the absence of endogenous *Pnky* (see next section: Quantitative PCR (qPCR)-based genotyping for BAC*Pnky*).

Primers for BAC*Pnky*:

BAC GT F: CACCTGCTACCTGATATAGGA

BAC GT R: CAGCAGTAATAGCAAGAGCA

Reaction product: 416bp (contains BAC*Pnky*). No amplification in the absence of BAC^{Pnky}.

Quantitative PCR (qPCR)-based genotyping for BAC^{Pnky}

Because BAC^{Pnky} contains unmodified *Pnky*, it is indistinguishable from *Pnky*+ in standard PCR-based genotyping. Therefore, certain genetic crosses required the use of qPCR to determine the endogenous *Pnky* alleles. This was done using 25ng of sample DNA (prepared as described above), along with 4µL SYBR green (Roche) and 2µL of

primer mix (with each primer at 5μM) per 8μL qPCR reaction. Reactions were amplified on a LightCycler 480 II (Roche) using standard conditions. To quantify the number of copies of particular endogenous *Pnky* alleles, the ΔΔCt method was used: a control genomic region was used to normalize for DNA content per reaction, and multiple samples with known endogenous *Pnky* alleles were used to compare to unknown samples.

Primers for control genomic region:

Ctrl qPCR GT F: TGGTCGTTCTACAGGCCTTC

Ctrl qPCR GT R: GGACCGGTGACAGAGAACTG

Primers for *Pnky*null allele:

Pnky qPCR GT F: AGTTGGTCGTCCGCGTACGGTAC

*Pnky*null qPCR GT R (same as *Pnky* GT R1):

TCAGGGACAAAGAACCAAAACGAGC

Product: 97bp from *Pnky*null allele. No amplification from other *Pnky* alleles or BAC*Pnky*.

Primers for *Pnky*F allele:

Pnky qPCR GT F: AGTTGGTCGTCCGCGTACGGTAC

*Pnky*F qPCR GT R: CCGGATCTTTCCTTTACCCGCAATAAC

Product: 228bp from *Pnky*F allele. No amplification from other *Pnky* alleles or BAC*Pnky*.

BrdU administration

Mice were administered 5-bromo-2'-deoxyuridine (BrdU, Millipore Sigma) reconstituted in sterile PBS through intraperitoneal injection, at a dose of 50mg BrdU per kg of mouse weight.

Tissue/cell preparation

Embryonic brain samples at E13.5 were fixed in 4% PFA as whole heads, up to overnight (O/N) at 4°C. For postnatal brain samples, transcardiac perfusion was performed on with phosphate buffered saline (PBS) followed by 4% PFA. The brains were then dissected out of the skull and additionally fixed in 4% PFA O/N at 4°C.

Cryo-sectioning

All specimens were rinsed in PBS and then cryoprotected with 30% sucrose in PBS. Cryoprotected samples were then equilibrated in a 1:1 mixture of 30% sucrose and Tissue-Tek Optimal Cutting Temperature (OCT) (Thermo Fisher Scientific) for 1.5 hour (hr) at 4°C, then frozen in a fresh batch of the same mixture using dry ice. Frozen blocks were equilibrated in the cryostat at -23°C for at least 3 hrs prior to sectioning. Sections (12-14μm thick) were collected on Superfrost Plus Microscope Slides (Thermo Fisher Scientific) and stored at -80°C. Prior to IHC, tissue slides were rinsed in PBS with rotation for 10 minutes (min) at room temperature (RT) to remove sucrose/OCT.

Immunohistochemistry (IHC)

IHC was performed using blocking buffer consisting of PBS with 1% BSA (Millipore Sigma), 0.3M glycine (Thermo Fisher Scientific), 0.3% TritonX100 (Millipore Sigma), and either 10% normal goat serum or 10% normal donkey serum (Jackson ImmunoResearch Laboratories).

IHC was performed as follows:

1) Blocking: tissue sections were incubated in blocking buffer for 1hrs (IHC) at RT.
2) Primary antibodies: incubated in primary antibodies diluted in blocking buffer O/N or 48 hours at 4°C (IHC).

3) Wash 1: washed 3 times in PBS at RT for 10 (IHC).

4) Secondary antibodies: incubated in secondary antibodies (Alexa Fluor antibodies from Thermo Fisher Scientific, 1:500) and DAPI (Thermo Fisher Scientific, 1:1000) diluted in blocking buffer for 30 min at RT (both IHC and ICC).

5) Wash 2: same as wash 1 above.

6) Mounting coverslips: slides were mounted with coverslips using Aqua Poly/Mount (Polysciences).

Antigen Retrieval

For certain antibodies (see below), antigen retrieval was performed using 10mM sodium citrate (pH 6.0) prior to IHC. Slides were incubated horizontally with 500µL of sodium citrate on top of the tissue for 2-3 min at RT. This was replaced with fresh sodium citrate, and the slides were moved to a pre-heated vegetable steamer. After 15 min, the slides were removed from the steamer and allowed to cool for 2-3 min at RT. The sodium citrate was then dumped off of the tissue and the slides were rinsed in PBS.

Primary antibodies for IHC/ICC

Please see Key Resources Table for antibody specifications. Antibodies were used as follows: **Tuj1**: diluted 1:1000 for ICC. **POU3F2**: diluted 1:250 for IHC (performed antigen retrieval for tissue of all ages). **CTIP2**: diluted 1:500 for IHC (performed antigen retrieval for postnatal tissue). **pH3**: diluted 1:400 for IHC. **pVim**: diluted 1:500 for IHC. **CUX1**: diluted 1:500 for IHC (performed antigen retrieval for postnatal tissue). **BrdU**: diluted 1:200 for IHC (performed antigen retrieval for tissue of all ages). **tdTomato** (Takara or Sicgen): diluted 1:500 for IHC or ICC (does not work after antigen retrieval, see below).

IHC for tdTomato in combination with antigen retrieval IHC for tdTomato was performed as described above, then the tissue was re-fixed with 4% PFA for 45 minutes at RT. Slides were rinsed in PBS, then antigen retrieval was performed followed by IHC for the other antigens as described above.

Microscopy and image analysis

Samples were imaged using Leica TCS SP5 X confocal, Leica DMi8, Leica DMI4000 B,

and Keyence BZ-X710 inverted microscopes. For tissue samples that had received *in utero* injection of Ad:Cre, the contralateral hemisphere was analyzed whenever possible. For all other tissue samples, both hemispheres were analyzed from 2-4 non-adjacent regions. All image analysis and quantification was performed using Fiji (Schindelin et al., 2012). To quantify Tuj1+ area (**Figures 3.4H-I**), the “threshold” and “measure” functions of Fiji were used.

Figure preparation

Figures were prepared using Photoshop and Illustrator (Adobe) and Prism (GraphPad).

Quantification and Statistical Analysis

All *in vivo* quantifications were normalized to littermate controls. For *in vivo* BAC rescue experiments, there was a low probability of obtaining all of the relevant experimental genotypes (*Pnky*+/+, *Pnky*null/null, and *Pnky*null/null;BAC*Pnky*) within the same litter. Therefore, I used one set of crosses to analyze *Pnky*+/+ and *Pnky*null/null littermates as one group, and another set of crosses to analyze *Pnky*null/null and *Pnky*null/null;BAC*Pnky* littermates as a separate group. To compare phenotypes

between these two groups, I normalized results to the genotype common to both (*Pnky*null/null), as shown in (**Figure 3.S4D**). For ICC quantification, technical triplicate wells of each genotype and treatment combination were analyzed. All cultures were normalized to their own treatment control. The statistical details of each experiment can be found in the relevant figure legends, with additional details for RNA-seq experiments described below.

Chapter 3: Histone lysine demethylase JMJD3/KDM6B is required for the establishment and maintenance of neural stem cells in hippocampus

Summary

JMJD3 is a chromatin regulator with histone demethylase activity that is critical for the activation of gene expression. Even though JMJD3 is involved in numerous cellular processes, the function of JMJD3 in the long-term maintenance of NSCs during development is poorly understood. NSCs in the hippocampal dentate gyrus (DG) generate new granule neurons throughout life, and defects in DG neurogenesis are associated with cognitive and behavioral problems. Here, I show that JMJD3 is required for both the establishment and maintenance of NSCs in the hippocampus. Conditional deletion of *Jmjd3* in results in precocious neuronal differentiation, resulting in reduced numbers of DG NSCs and impaired NSC niche establishment. Using the single-cell RNA technology, I found that a stem cell maintenance signature is disrupted in *Jmjd3*-deleted NSCs. When JMJD3 deletion was targeted adult NSC in a cell-autonomous manner, NSCs precociously produced neurons at the expense of the NSC pool. Thus, JMJD3 is required for the establishment and maintenance of the hippocampal NSC niche.

Introduction

The DG of the hippocampus is one of two brain regions where NSCs are established after embryonic development. This structure is also fundamentally important for certain forms of learning, memory and emotional behavior (Deng et al., 2010; Kempermann et al., 2015; Ming and Song, 2011). In the DG, NSCs are located in a subgranular zone (SGZ) where they give rise to excitatory granule neurons throughout life (Nicola et al., 2015). It was shown that genetic depletion NSCs in the hippocampus leads to cognitive defects. (Burghardt et al., 2012). This has been suggested to underlie human neurodevelopmental and cognitive disorders (Khacho et al., 2017; Li et al., 2018).

Recent studies have implicated mutations in dozens of chromatin regulators as causes of human neurodevelopmental and psychiatric disorders (De Rubeis et al., 2014; Ronan et al., 2013). Mutations in the chromatin regulator *JMJD3* (*KDM6B*) are autosomal recessive for intellectual disability (Najmabadi et al., 2011; Yavarna et al., 2015). Furthermore, *de novo* mutations in *JMJD3* are associated with autism spectrum disorder (ASD) (De Rubeis et al., 2014; Iossifov et al., 2014; Sanders et al., 2015). These genetic data indicate that JMJD3 plays key roles in the development of the brain. JMJD3 is a chromatin regulator that has demethylase activity for histone 3 lysine 27 trimethylation (H3K27me3) (Agger et al., 2007; De Santa et al., 2007), a chromatin modification that correlates with transcriptional repression. Consequentially, loss of JMJD3 results in a failure to remove the H3K27me3 repressive mark; therefore, genes targeted by JMJD3 fail to get de-repressed and are not transcribed.

In mouse ESCs, *Jmjd3* is essential for neural lineage commitment (Burgold et al., 2008), and the expression of *Jmjd3* is regulated during mouse forebrain development (Jepsen et al., 2007). Knockdown of *Jmjd3* in the chicken embryonic spinal cord (Akizu et al., 2010) and mouse retina (Iida et al., 2014) indicate critical roles for this chromatin regulator in the developing central nervous system (CNS). In the peripheral nervous system, *Jmjd3* is essential for nerve injury and recovery by modulating the proliferation of Schwann cells (Gomez-Sanchez et a., 2013). *Jmjd3* has been also implicated in the modulation of neuro-inflammation and neurodegenerative diseases (Burchfield et al., 2016) where it regulates the balance of microglial cells between the pro-inflammatory or anti-inflammatory type (Tang et al., 2014). Dynamic roles of *Jmjd3* throughout various regions in nervous system suggest that its function is context dependent.

Interestingly, in human patients with ASD, MRI studies reveal that the hippocampus sub-region containing the DG is also smaller compared to controls (Saitoh et al., 2001). Understanding the roles that JMJD3 play in the development of structure critical to learning and memory may inform our understandings of certain cognitive disorders. Whether JMJD3 is required for the establishment of the NSC niche in hippocampus, and whether such deficiency can cause a defect in long-term NSC function was not known. Using multiple genetic approaches combined with single cell RNA-sequencing technology, I investigated the role of JMJD3 in the DG NSC niche. My data indicate that *Jmjd3* is essential for not only the establishment of NSCs in the DG but also for their long-term maintenance within this niche, allowing for the generation of neurons throughout life. More broadly, my findings provide insight into how defective

JMJD3 activity can result in developmental CNS problems that may contribute to cognitive disorders.

Results

Jmjd3 expression in the developing hippocampal dentate gyrus

In the subgranular zone (SGZ) of the adult hippocampal DG (**Figure 3.1A**), NSCs produce intermediate-progenitor cells (IPCs), which give rise to neuroblasts that migrate into the granule cell layer (GCL). In the GCL, neuroblasts terminally differentiate to become excitatory granule neurons (**Figure 3.1B**). At postnatal day 21 (P21) in the DG, nuclear JMJD3 can be detected in virtually all DG cells including the SGZ NSCs (**Figure 3.1C, Figure 3.S1F**). The SGZ NSC population arises from embryonic radial glial cells – the NSCs of the developing brain – located in a region of the ventricular zone called the dentate epithelium (DNE, **Figure 3.S1A**) (Rolando and Taylor, 2014). *In situ* hybridization (ISH) revealed *Jmjd3* expression in the E16.5 DE as well as the dentate migratory stream, which contains embryonic NSCs *en route* to the developing DG (**Figure 3.S1B-C**). By the end of the first postnatal week, NSCs localize to the inner layer of the DG (Nicola et al., 2015). *Jmjd3* was detected throughout the DG at both P1 and P7 (**Figure 3.S1D-E**). As postnatal development continues, these NSCs consolidate into the SGZ. *Jmjd3* expression remained prominent in the SGZ and GCL at P21 (**Figure 3.S1A, S1F**). These data indicate that *Jmjd3* is expressed in the developing DG during the time when the SGZ NSC population becomes established.

Jmjd3 deletion leads to depletion of NSCs in the hippocampus

To investigate the role of *Jmjd3* in DG development, I targeted *Jmjd3*-deletion to the developing hippocampus by crossing mice expressing Cre under the control of the

human glial fibrillary acidic protein promoter (*hGFAP-Cre*) with mice carrying conditional knockout alleles of *Jmjd3* (*Jmjd3$^{F/F}$*) (**Figure 3.S1B**) (Iwamori et al., 2013; Park et al., 2014). *hGFAP*-Cre is expressed in the hippocampal ventricular zone and results in efficient recombination in cells of the DNE and developing DG by E16.5 (Han et al., 2008). As expected, the DNE and DG of *hGFAP-Cre;Jmjd3$^{F/F}$* mice lacked *Jmjd3* transcripts as detected by ISH probes to the deleted genomic region (**Figure 3.S1C', S1D'**). *hGFAP-Cre;Jmjd3$^{F/F}$* mice and their littermate controls (*Jmjd3$^{F/+}$, Jmjd3$^{F/F}$* and *hGFAP-Cre;Jmjd3$^{F/+}$*) were born at the expected Mendelian ratios and were similar in overall size, weight and survival throughout postnatal and adult life (Park et al., 2014).

In adult (P60) *hGFAP-Cre;Jmjd3$^{F/F}$* mice, the dentate gyrus was very small and hypocellular as compared to littermate controls (**Figure 3.1D-D'**). SGZ NSCs express NESTIN and GFAP and extend radial processes into the granule cell layer (GCL) (**Figure 3.1B**) (Kempermann et al., 2015). In wild-type adult mice, I observed NESTIN+, GFAP+ cells in the SGZ with radial processes, consistent with the presence of SGZ NSCs (**Figure 3.1E, 1F, 1G**). In stark contrast, in *hGFAP-Cre;Jmjd3$^{F/F}$* mice, I could not identify any cells with these morphological and immunohistochemical characteristics, suggesting the absence of SGZ NSCs in utant mice (**Figure 3.1E', 1F', 1G'**). SOX2 is expressed in SGZ NSCs, and SOX2+ cells were also severely reduced in the disorganized and small SGZ of *hGFAP-Cre;Jmjd3$^{F/F}$* mice (**Figure 3.1H-H'**). Young neuroblasts express Doublecortin (DCX). While many DCX+ cells were observed in the DG of control mice essentially no DCX+ cells were detected in the DG of *hGFAP-Cre;Jmjd3$^{F/F}$* mice (data now shown), consistent with the absence of SGZ NSCs and a lack of adult neurogenesis. Thus, without *Jmjd3*, the adult DG lacks SGZ NSCs, and

proper adult neurogenesis fails.

Jmjd3 is required for postnatal dentate gyrus development

Shortly after birth, the DG continues to grow and by P21 it is considered to be fully developed (Nicola et al., 2015). At P0, the developing DG in *hGFAP-Cre;Jmjd3$^{F/F}$* mice was similar to littermate control mice. While the DG in control mice continued to grow, however, exhibiting a progressive increase in its blade length from P0 to P21, the DG in *hGFAP-Cre;Jmjd3$^{F/F}$* mice failed to increase in size (**Figure 3.1I**). The failure of progressive DG growth correlated with a reduction in NSCs throughout postnatal development. Consistent with this, the number of SOX2+ PAX6+ DG precursors was already at P0 and this reduction persisted at P7. By P15 and P21, the reduction in the number of NSCs, as defined by SOX2+ PAX6+ GFAP+ expression and accompanying radial processes was still observed in the SGZ of *Jmjd3*-deleted brain (**Figure 3.1K**).

The reduced SGZ NSC population in *hGFAP-Cre;Jmjd3$^{F/F}$* mice could potentially result from increased cell death. However, I did not observe an increase in activated Caspase3+ cells in the DG of P0 and P7 *hGFAP-Cre;Jmjd3$^{F/F}$* mice (**Figure 3.S2A-S2D**), suggesting that increased apoptosis did not account for the DG phenotype observed. Similarly, previous studies have not observed increased cell death with *Jmjd3* deficiency in mouse postnatal subventricular zone NSCs (Park et al., 2014), retinal progenitors (Iida et al., 2014), developing medulla (Burgold et al., 2012) and ESCs (Burgold et al., 2008). Taken together, *Jmjd3*-deletion leads to impaired DG growth and reduction in NSCs, and these phenotypes are not due to increased apoptosis.

Without *Jmjd3*, DG precursor cells undergo precocious neuronal differentiation

To investigate the phenotype of *Jmjd3*-deletion in precursor cells, I performed 5-bromo-2-deoxyuridine (BrdU) birthdating experiments. At E15.5, the number of SOX2+ neural progenitor cells (NPCs), as well as the number of proliferative cells was very similar between *hGFAP-Cre;Jmjd3$^{F/F}$* and control mice (**Figure 3.S3A-C**). I pulse-labeled E15.5 embryos (*Jmjd3*-deleted vs control) by injecting pregnant mice with BrdU and subsequently analyzed the DG for BrdU+ cells 2 days and 4 days post injection (dpi) (**Figure 3.2A**). In the wild type DG at E17.5 (2dpi), DG precursor cells start to migrate away from the DNE, forming dentate migratory stream (DMS) toward the final location where the DG blades will develop (**Figure 3.2B**). I quantified the number of BrdU+, SOX2+ neural precursor cells and BrdU+, TBR2+ intermediate progenitor cells (IPCs) in the developing DG. In *Jmjd3*-deleted brains, the number of BrdU+, TBR2+ IPCs was increased compared to littermate controls. This increase was observed in both areas of migrating precursor cells and developing DG blade (**Figure 3.2C-D**). The increase in the number of BrdU+, TBR2+ IPCs was accompanied by a reduced number of BrdU+, SOX2+ precursor cells in the developing DG blade (**Figure 3.2D**). At P.5 (4dpi), the increased number of IPCs differentiated into BrdU+, TBR2+, PROX1+ young neuroblasts, suggesting that excess BrdU+ TBR2+ IPCs are able to further differentiate down the neurogenic lineage in *Jmjd3*-deleted brain (**Figure 3.2E-F**). These increases in IPCs and neuroblasts were accompanied by a reduced numbers of BrdU+ SOX2+ neural progenitor cells. Thus, without *Jmjd3*, SGZ precursor cells precociously differentiate into neurons, resulting in reduced of SGZ NSCs in *hGFAP-Cre;Jmjd3$^{F/F}$* and failure to properly establish the SGZ (**Figure 3.1K**).

Single-cell RNA sequencing resolves the cellular heterogeneity in the developing DG

The developing DG consists of multiple cell types. Since bulk tissue RNA-seq analysis would only reveal the "merged" transcriptome of these cell types and states, I performed droplet-based single-cell RNA sequencing (scRNA-seq) on micro-dissected DG from P2 control and *hGFAP-Cre;Jmjd3*$^{F/F}$ mice (n=2 per group) (**Figure 3.3A**). ScRNA-seq would also enable the discovery of specific neural cell types as well as important aspects of their cell state. (*e.g.,* stemness, quiescence and proliferation). Unsupervised clustering of cells with high-quality transcriptomes (3163 and 2077 DG cells from control and hGFAP-Cre*;Jmjd3*$^{F/F}$ animals, respectively) revealed 16 transcriptionally distinct cell clusters (**Figure 3.S4A**). *Jmjd3*-deletion was confirmed by examining read alignment data (not shown). Major cell types were identified based on expression levels of well-characterized marker genes, with the most abundant cell types coming from the hippocampal neurogenic lineage (NSCs, IPCs, and granule neurons) (**Figure 3.S4B**). I identified an "NSC cluster" (~13% of the population for both control and *Jmjd3*-deleted NSCs) based on co-expression of HOPX, SOX2, PAX6, and GFAP (**Figure 3.3B-C**). I also identified several other expected cell types, including inhibitory interneurons, microglia, endothelial cells, Cajal-Retzius cells, stromal cells, and oligodendrocyte precursor cells (**Figure 3.S4C**). Analysis of cell clusters based on genotypes (*hGFAP-Cre;Jmjd3*$^{F/F}$ vs control) revealed wild-type and *Jmjd3*-deleted cells to be present in all clusters (**Figure 3.S4D**).

Jmjd3-deleted DG NSCs have an impaired NSC maintenance gene signature

To gain insight into the *Jmjd3*-dependent transcriptome of DG NSCs, I performed differential gene expression analysis between NSCs from hGFAP-Cre*;Jmjd3*$^{F/F}$ mice and littermate controls. I identified 339 statistically significant differentially expressed (DE) genes (119 and 218, down-regulated and up-regulated in *Jmjd3*-deleted NSCs, respectively) at a Bonferroni adjusted *p*-value <0.05) (**Figure 3.3D**). These DE genes showed statistically significant enrichment for genes previously reported to be important in hippocampal NSCs (**Figure 3.S4E**) (Shin et al., 2015). To investigate whether JMJD3 localizes to the promoters of these DE genes, I analyzed published JMJD3 ChIP-seq data from NSCs derived from E12.5 mouse cortex (Fueyo et al., 2018). Of the 339 DE genes in *Jmjd3*-deleted NSCs, 185 (54.6%) exhibited JMJD3 enrichment (**Figure 3.S4F**). I did not find differences in the expression of the other known H3K27me3 specific demethylase *Utx (Kdm6a)* or the primary H3K27me3 methyltransferase *Ezh2*.

Jmjd3-deleted NSCs expressed significantly lower levels of inhibitors of DNA binding and cell differentiation (*Id*) genes (**Figure 3.3E**). *Id* family members, including *Id1, Id2,* and *Id3* have been previously shown to be essential for NSC maintenance, with knockout or knockdown causing precocious neuronal differentiation (Niola et al., 2012). Other genes implicated in NSC maintenance such as insulin growth factor binding protein 2 (*Igfbp2*) were also down-regulated (**Figure 3.3E**). *Igfbp2* promotes self-renewal of NSCs, *Igfbp2* knockdown results in precocious neuronal differentiation (Shen et al., 2018). Milk fat globule-EGF factor (*Mfge8*) and glutamate receptor (*Grm5*) were also both down-regulated in *Jmjd3*-deleted NSCs (**Figure 3.3E**). Low expression level of either of these genes leads to NSC depletion due to premature neuronal

differentiation (Xiao et al., 2013). *Mfge8* is also essential for NSC maintenance, as deletion of *Mfge8* leads to depletion of NSCs due to due to their overactivation (Zhou et al., 2018).

Fatty acid degradation pathways and glycolysis are molecular signatures enriched in hippocampal NSCs (Shin et al, 2013). In my analyses, numerous genes involved in glycolysis (*Aldoa, Ldha, Ldhb*), fatty acid degradation (*Acsbg1*), and drug metabolism (*Hspd1, Pgk1, Pgam1, Eno1, Gpi1*) were down-regulated in *Jmjd3*-deleted NSCs (**Figure 3.3E**). Gene ontology analysis of down-regulated DE genes revealed statistically significant enrichment of terms related to glycolysis and other metabolic processes (*e.g.,* "Glucogenesis," and "ATP generation") (**Figure 3.3F**) (Shin et al., 2015). Interestingly genes that have been reported to promote neuronal differentiation, such as Wnt signaling pathway genes (*Wnt5a, Fzd, Wls*), were upregulated. Our findings show that scRNA-seq can be used not only for studying subpopulations of cells in a complex tissue, but also for studying study cell-specific phenotypes following genetic manipulation. Together, these data suggest that the stem cell maintenance molecular signature was disrupted in *Jmjd3*-deleted NSCs, changes that could lead to precocious neuronal differentiation.

JMJD3 is required for the maintenance of adult hippocampal NSCs

My postnatal analysis of NSCs in hGFAP-Cre;*Jmjd3*$^{F/F}$ mice does not distinguish between the roles of JMJD3 in the establishment and maintenance of the NSC population. Furthermore, since *hGFAP-Cre* deleted *Jmjd3* in most DG precursors starting E13.5, non cell-autonomous effects may have contributed to defective NSC maintenance at P2. To investigate whether *Jmjd3* plays a cell-autonomous role in DG

NSC maintenance, I used a tamoxifen (TAM)-inducible Nestin-Cre^{ERT2} (Lagace et al., 2007) strain to acutely induce *Jmjd3* deletion in adult mice. To follow the fate of cells that had undergone recombination, I used the Ai14 Cre-reporter transgene, which express tdTomato after excision of a “floxed-stop” cassette (Madisen et al., 2009). Nestin-Cre^{ERT2}; *Jmjd3$^{F/F}$*;Ai14 and Nestin-Cre^{ERT2} ;*Jmjd3$^{F/+}$*;Ai14 (littermate controls) mice were administered tamoxifen (TAM) for 5 days starting at P60. At 1 day post last injection (dpi) of TAM administration, the total number of tdTomato+ cells in the DG of Nestin-Cre^{ERT2}: Jmjd3$^{F/F}$ mice was similar compared to littermate control suggesting that deletion of *Jmjd3* does not have an impact on labeling efficiency (Figure 3.4A). However, at 10dpi, I observed a 28% increase in tdTomato+ Tbr2+ intermediate progenitors and 43% increase in tdTomato+ DCX+ Tbr2+ neuroblasts in Nestin-Cre^{ERT2};Jmjd3$^{F/F}$;Ai14 mice, suggesting that these animals exhibited precocious neuronal differentiation (**Figure 3.4C-D**). To evaluate whether this precocious neuronal differentiation results in depletion of DG NSCs (tdTomato+ SOX2+ with radial processes), I analyzed animals 30dpi. As expected, I observed a decrease in the number of DG NSCs (~25%) in Nestin-Cre^{ERT2};Jmjd3$^{F/F}$;Ai14 mice compared to littermate controls (**Figure 3.4E-F**). These results suggest that *Jmjd3* is required for NSCs maintenance, as *Jmjd3* loss results in precocious neuronal differentiation similar to its earlier role in the establishment of SGZ NSCs.

Discussion

Chromatin regulators have been implicated in essential processes throughout neural development, and mutations in a diverse array of chromatin regulators are associated with human neurodevelopmental and psychiatric disorders (Pedersen and Helin, 2010). A key unanswered question about the function of chromatin regulators is whether they play a cell-intrinsic role in the establishment and long-term maintenance of NSCs *in vivo.* Using a combination of transgenic mice, I have shown that *Jmjd3* regulates long-term NSC functions cell-intrinsically in the hippocampal dentate gyrus. By targeting *Jmjd3*-deletion to NSCs in the embryonic and adult brain, I found that *Jmjd3* regulates NSC function such that loss of *Jmjd3* leads to precocious neuronal production at the expense of NSCs. Single cell RNA-sequencing revealed a disruption of genes involved in maintaining NSC identity, consistent with the idea that *Jmjd3* is required for NSC maintenance.

Jmjd3-deletion results in depletion of NSCs due to failure to establish the NSC population as well as impaired maintenance of the stem cell pool. The phenotyuupe of precocious neuronal differentiation suggests that *Jmjd3* acts like a cell-intrinsic clock, deciding the timing of neuronal differentiation vs. self-renewing cell divisions. Interestingly, the function of *Jmjd3* in hippocampal NSCs appears to be distinct as compared to a previous study in the other adult NSC niche, the V-SVZ (Park et al., 2014). In V-SVZ NSCs, *Jmjd3* is required to activate essential neurogenic gene expression via interactions at enhancer and promoter regions. *Jmjd3*-deleted NSCs in the V-SVZ seem to be “stalled” in the precursor state, resulting in a severe reduction in olfactory bulb neurogenesis. In the adult V-SVZ of *Jmjd3*-deleted brains, accumulation

of NSCs is observed with no evidence of defects in cell proliferation nor an increase in cell death (Park et al., 2014).

Other studies suggest that *Jmjd3* plays distinct roles in a cell type-specific manner. In mesenchymal stem cells (MSC), the function of *Jmjd3* is related to preferential lineage specification. *Jmjd3* preferentially regulates adoption of an osteogenic lineage over the adipogenic lineage through its interaction with the *Runx2* transcription factor (Zhang et al., 2015). During lung development, *Jmjd3* functions in a stage-, and tissue-specific manner. Depending on the timing and localization of *Jmjd3* deletion, embryos can either survive or die shortly after birth due to respiratory problems (Li et al., 2014). *Jmjd3* also regulates cellular programming, acting as a negative regulator of reprogramming in the generation of induced pluripotent stem cells (iPSCs) (Zhao et al., 2013). During development, *Jmjd3* is required for a lineage specification of embryonic stem cells. Therefore, when somatic cells are driven to undergo cellular reprogramming to pluripotency, *Jmjd3* may serve as a roadblock (Burchfield et al., 2015). These data, taken together with our studies, illustrate that *Jmjd3* can regulate stem cell functions in various ways and its precise function and mechanism of action depends on context (e.g. cell and tissue type differences).

One major unanswered question in the chromatin biology field is how these enzymes get recruited to specific regions in the genome. The dynamic nature of *Jmjd3* functions may be attributed to different binding or interaction partners, whose expression is cell or tissue-specific. JMJD3 itself does not have a DNA binding domain, indicating that in order to exert its functions on gene expression, it likely has to interact with other proteins or complexes to be recruited. It has been shown that *Jmjd3* can

interact with transcription factors (e.g. SMAD, NKX2.1, TBX3, and others) (Estaras et al., 2012; Akizu et al., 2010; Kartikasari et al; 2013), chromatin remodelers (e.g. CHD8, BAF) (Miller at el., 2010; Fueyo et al., 2018; Narayanan et al., 2015), and even lncRNAs (*HOTAIR* and *ARHGAP27P1*) (Xia and Yao et al., 2017; Zhang et al., 2019). In V-SVZ NSCs, it is thought that *Jmjd3* interacts with ASCL1, proneural transcription factor (Aydine et al., 2019). Motif analysis has suggested that the enhancer element of DLX2 that is bound by JMJD3 is also bound by ASCL1 (Liu et al., 2017; Lindtner et al., 2019). Future studies aimed at determining JMJD3's binding partners will provide critical insights into how one histone modifying enzyme can carry out multiple functions in a context-dependent manner.

Single cell RNA technology shed additional light on our *Jmjd3* mutant mouse phenotype, revealing defects in a stem cell maintenance gene expression signature upon loss of *Jmjd3* in the hippocampal DG. For instance, Inhibitors of DNA binding and cell differentiation (*Id*) genes, well known for their essential roles in stem cell maintenance and promote self-renewal, are down-regulated in *Jmjd3*-deleted NSCs. Knockout or knockdown of *Id* genes leads to precocious differentiation, and deletion of *Id1, Id2*, and *Id3* in NSCs results in neuronal differentiation accompanied by depletion of embryonic NSCs, similar to what I observe in *Jmjd3* mutant brains (Niola et al., 2012). Based on published Jmjd3 ChIP-seq data, all of *Id* genes exhibit *Jmjd3* enrichment at their promoter regions, suggesting that *Id* genes may be down-stream targets of *Jmjd3* (Fueyo et al., 2018).

Gene-ontology analysis of disrupted genes in *Jmjd3*-deleted NSCs also revealed an enrichment for genes involved in glycolysis and metabolism. While it is possible that

this change may be due to indirect effects of *Jmjd3*-deletion, the majority of these genes exhibit JMJD3-enrichment at their promoter regions, further suggesting involvement of JMJD3 in the regulation of energy metabolism and glycolysis (Fueyo et al., 2018). To date, there has been some speculations regarding histone demethylases having an effect on metabolism, but direct evidence is lacking.

Aside from *Id* genes and metabolism genes, components of the WNT signaling pathway (*Wnt5a, Fzd, Wls*) are upregulated in *Jmjd3*-deleted NSCs. In the hippocampus, WNTs are secreted by NSCs, astrocytes, and granule neurons, and WNTs gave been shown to regulate NSC function in both cell-intrinsic and cell-extrinsic ways (Okamoto et al., 2011). Interestingly, *Wnt5a* has been shown to promote neuronal differentiation, which may at least in part explain the increased neuronal production observed in *Jmjd3*-deleted NSCs. *Jmjd3* may also be involved in regulating the Notch signaling pathway. Components of Notch signaling pathway, *Hes1* and *Notch2*, were differentially expressed in *Jmjd3*-deleted NSCs. Both genes exhibited *Jmjd3* enrichment at their promoter region (Fueyo et al., 2018). Interestingly, loss of the essential Notch components, *RBPJk* or *Jag1*, in embryonic NSCs leads to proliferation defects as well as precocious neuronal differentiation, resulting in NSC depletion (Imayoshi et al., 2010). Taken together, these data suggest that essential signaling pathways for stem cell function may be regulated through chromatin modifiers.

My results also suggest a potential neurodevelopmental link between *Jmjd3* mutations and aberrant hippocampal development and neurogenesis, providing new insights into how mutations in chromatin regulators may contribute to learning disorders and other neurological disorders. Given that *JMJD3* mutations have been causally

implicated in autosomal recessive forms of intellectual disability (Najmabadi et al., 2011; Yavarna et al., 2015), it is interesting to consider the possibility that the DG in these patients is abnormal in structure and deficient in adult neurogenesis. *De novo* mutations in *JMJD3* that likely cause haploinsufficiency have also been identified in patients with ASD (De Rubeis et al., 2014; Iossifov et al., 2014; Sanders et al., 2015). Interestingly, *Jmjd3*-heterozygote mice exhibited small (~10%) but statistically significant reduction in the size of the dentate gyrus (Figure 3.1J), hinting at potential dosage-dependent functions of *Jmjd3*. Using MRI, a previous study reported that patients with ASD had a smaller dentate gyrus (~13.5%) compared to healthy controls (Saitoh et al., 2001). I speculate that the increased complexity and duration of human brain development may increase vulnerability to *JMJD3* haploinsufficiency (*e.g.,* via reduced gene dosage, or monoallelic expression).

As NSCs in embryonic and adult brain differentiate, the rate at which neurons are produced must be precisely regulated. Correct timing of differentiation may be regulated by a cell-intrinsic clock, and dysfunction of this clock can lead to precocious neuronal differentiation at the expense of NSCs. Based on *in vivo* data presented above, *Jmjd3* is required to maintain NSCs by acting as a clock balancing between self-renewal vs differentiation during development. *Jmjd3*-dependent transcriptome analysis revealed a disruption of NSC maintenance gene expression signature that includes previously characterized regulators of NSC maintenance (WNT, NOTCH, and *Id* genes) (Arredondo et al., 2019; Imayoshi et al., 2010; Niola et al., 2012). Taken together, my data suggest that JMJD3, as a chromatin regulator, orchestrates a broad transcriptome required for NSC maintenance, modulating multiple signaling pathways simultaneously.

Figures and Tables

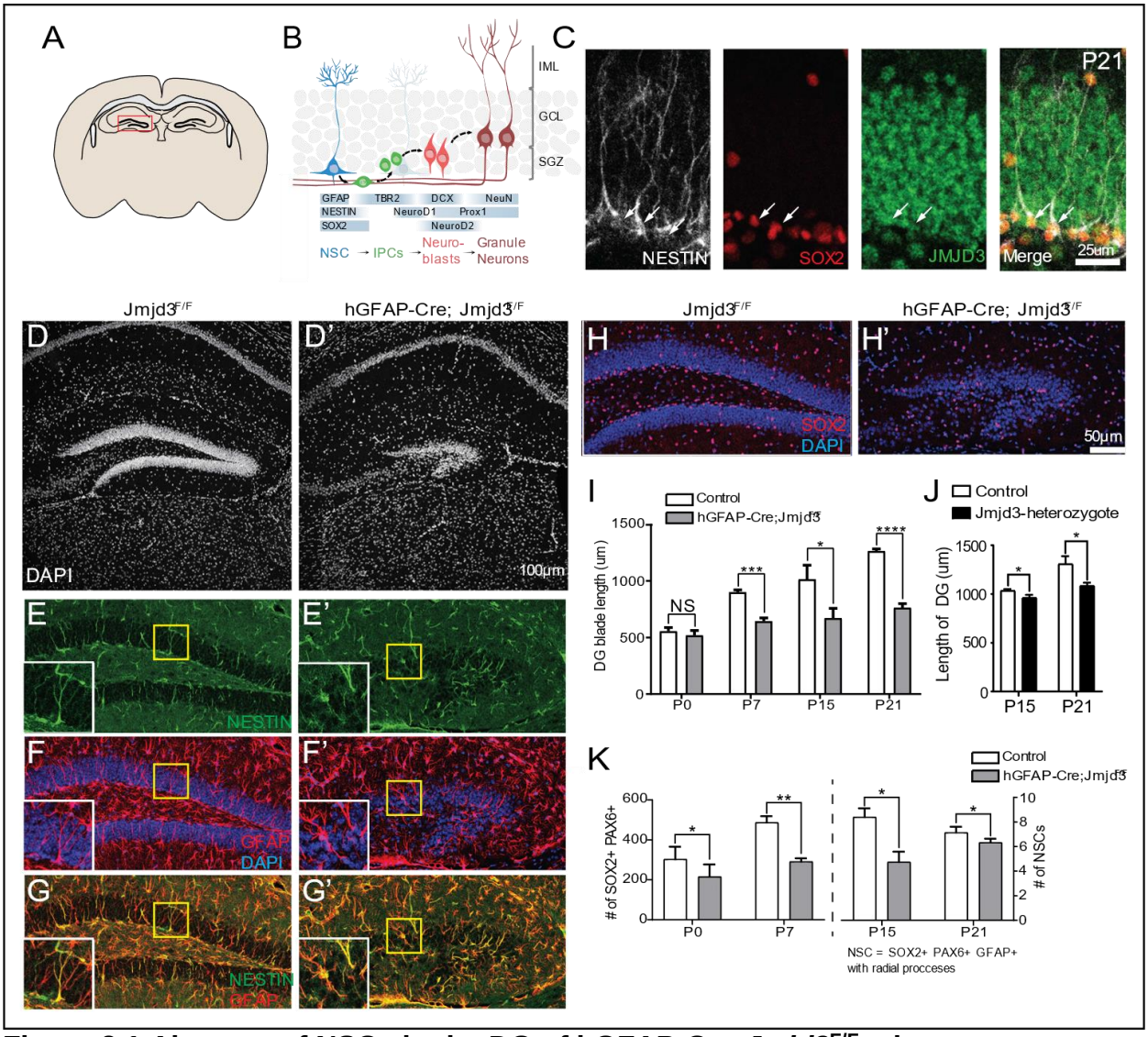

Figure 3.1 Absence of NSCs in the DG of hGFAP-Cre;*Jmjd3*$^{F/F}$ mice.
* = p < 0.05, ** = p < 0.01, *** = p < 0.0005, **** = p < 0.0001, NS = not significant
A, Schematic coronal section showing the dentate gyrus of the hippocampus; red box indicates regions shown in (B). **B,** Schematic illustration of neurogenesis in the DG. **C,** JMJD3 (green) expression in the DG of P21 mouse brains. Immunohistochemistry (IHC) is shown for SOX2 and NESTIN. **D-G'** IHC for NESTIN (green), GFAP (red), and DAPI (white or blue) in coronal DG sections of P60 control (D-G) and *hGFAP-Cre;Jmjd3*$^{F/F}$ mice (D'-G'). **H-H',** IHC for SOX2 (red) and DAPI (blue) in coronal DG sections of control (H) and *hGFAP-Cre;Jmjd3*$^{F/F}$ mice (H'). **I,** Quantification of length of the DG in control and *hGFAP-Cre;Jmjd3*$^{F/F}$ mice from P0 and P21 (n = 3 each), two-tailed unpaired t test. **J,** Quantification of length of the DG in control and *hGFAP-Cre;Jmjd3*$^{F/+}$ mice P15 and P21 (n = 3 each), two-tailed unpaired t test. K, Quantification of neural precursor/NSCs in control and *hGFAP-Cre;Jmjd3*$^{F/F}$ mice from P0 and P21 (n = 3 each), two-tailed unpaired t test.

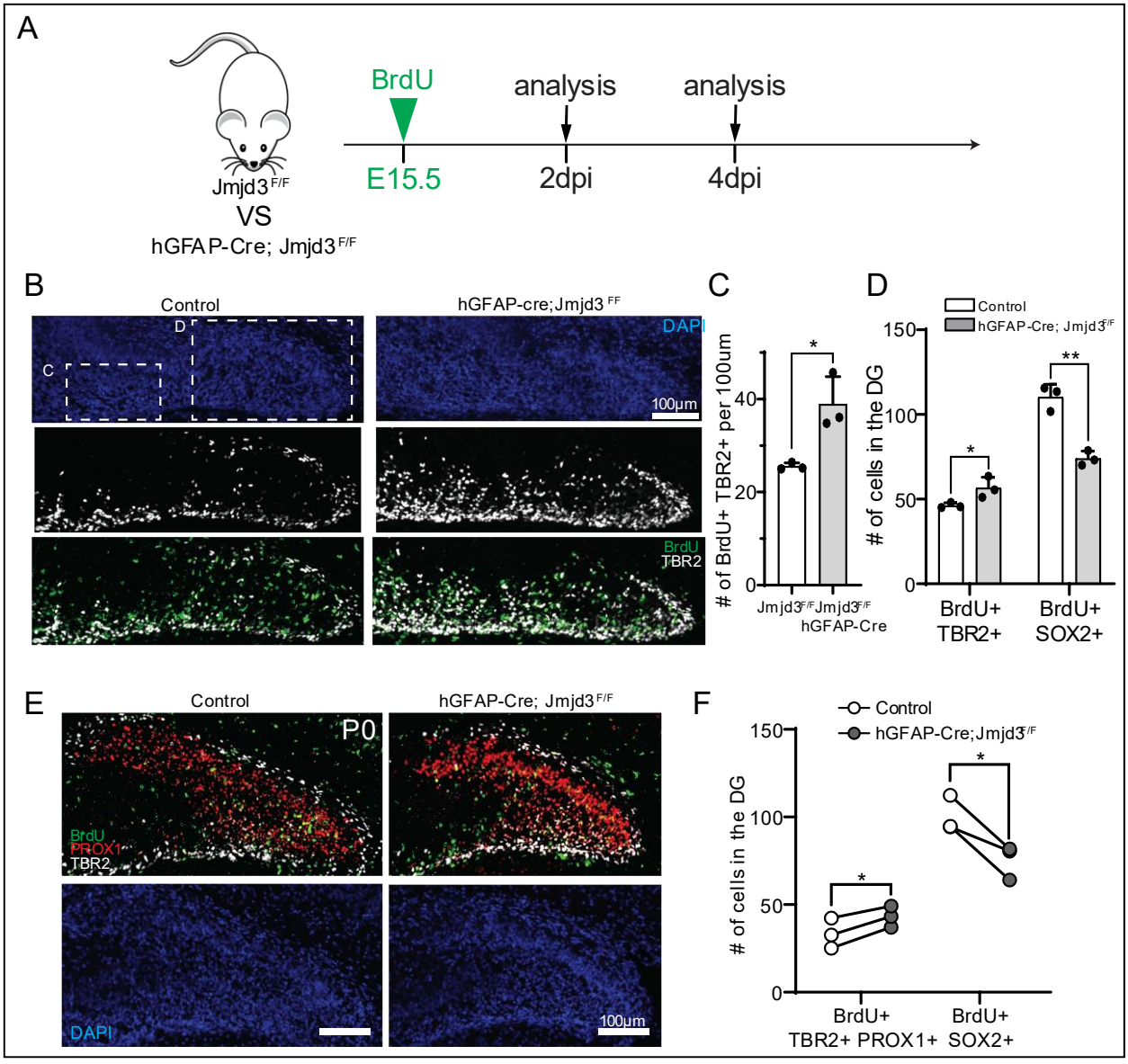

Figure 3.2 Precocious neuronal differentiation of SGZ precursors in hGFAP-Cre;*Jmjd3*$^{F/F}$ mice.

* = p < 0.05, ** = p < 0.01

A, Schematic of experimental design. **B,** IHC for BrdU (green) and TBR2 (white) with DAPI (blue) in coronal sections of developing DG in control and *hGFAP-Cre;Jmjd3*$^{F/F}$ mice at E17.5. **C,** Dentate migratory stream (DMS) and quantification of BrdU+ TBR2+ cells in dentate migratory stream, two-tailed unpaired t test. **D,** Quantification of BrdU+ TBR2+ and BrdU+ SOX2+ cells in coronal sections of DG in control and *hGFAP-Cre;Jmjd3*$^{F/F}$ mice, two-tailed unpaired t test. E, IHC for BrdU (green), PROX1 (red), TBR2 (white), and DAPI (blue) in coronal sections of DG in control and *hGFAP-Cre;Jmjd3*$^{F/F}$ mice at P0.5. **F,** Quantification of (E), two-tailed paired t test.

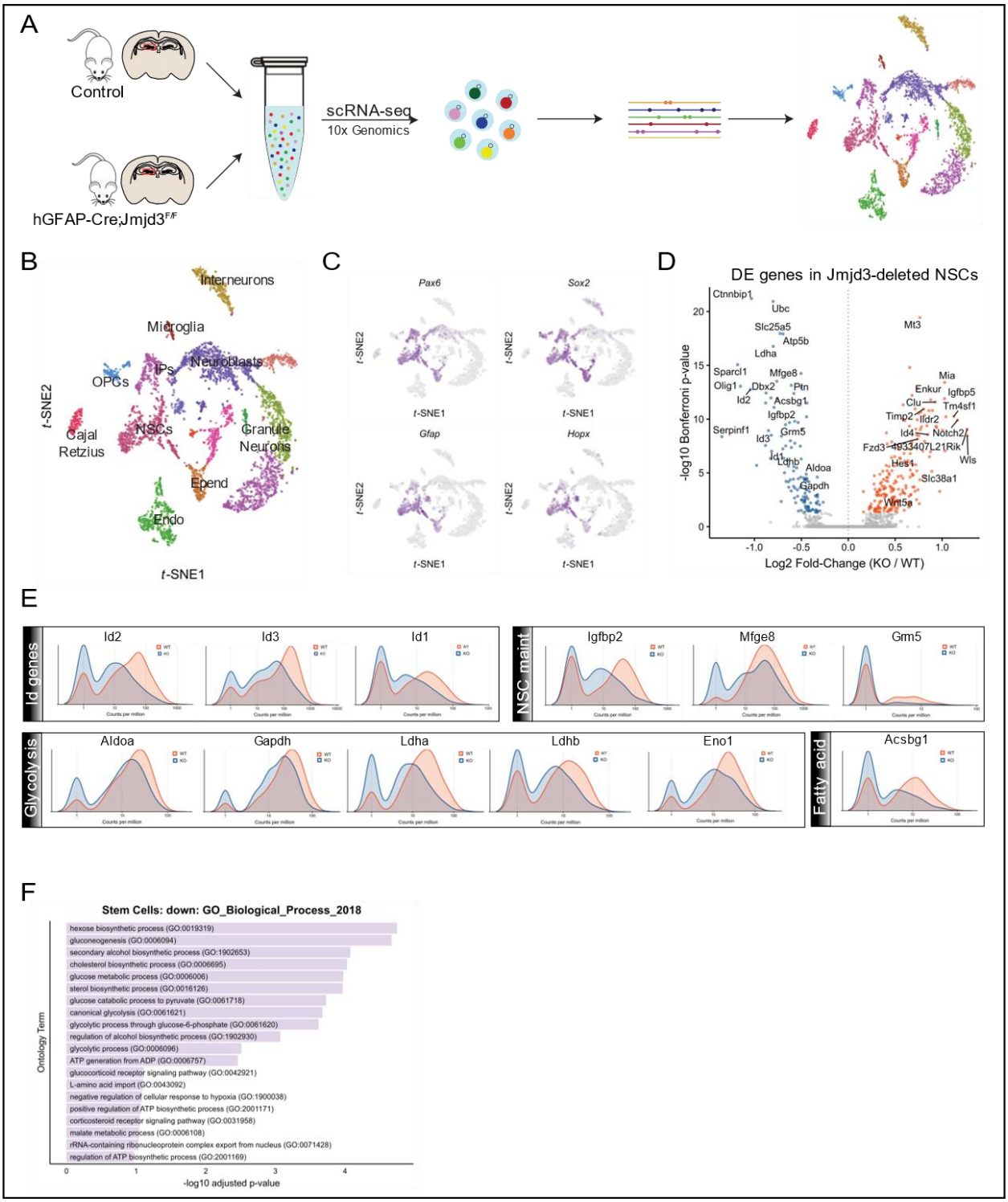

Figure 3.3 Single cell RNA-seq reveals disrupted stem cell maintenance gene signature in Jmjd3-deleted NSCs

A, Schematic of single cell RNA-seq experiment design. **B,** t-SNE plot of DG cells from hGFAP-Cre;*Jmjd3*$^{F/F}$ and control mice labeled with corresponding cell type identity. **C,** t-SNE plot of DG cells with specific marker expression. **D,** Volcano plot of differentially

expressed genes in NSCs. **E,** Density plot of down-regulated genes in Jmjd3-deleted NSCs comparing to control, genes are grouped into, Id genes, glycolysis pathway genes, fatty acid pathway, and previously identified “stem-cell maintenance genes”. **F,** Gene ontology terms identified for statistically significant down-regulated genes in Jmjd3-deleted NSCs.

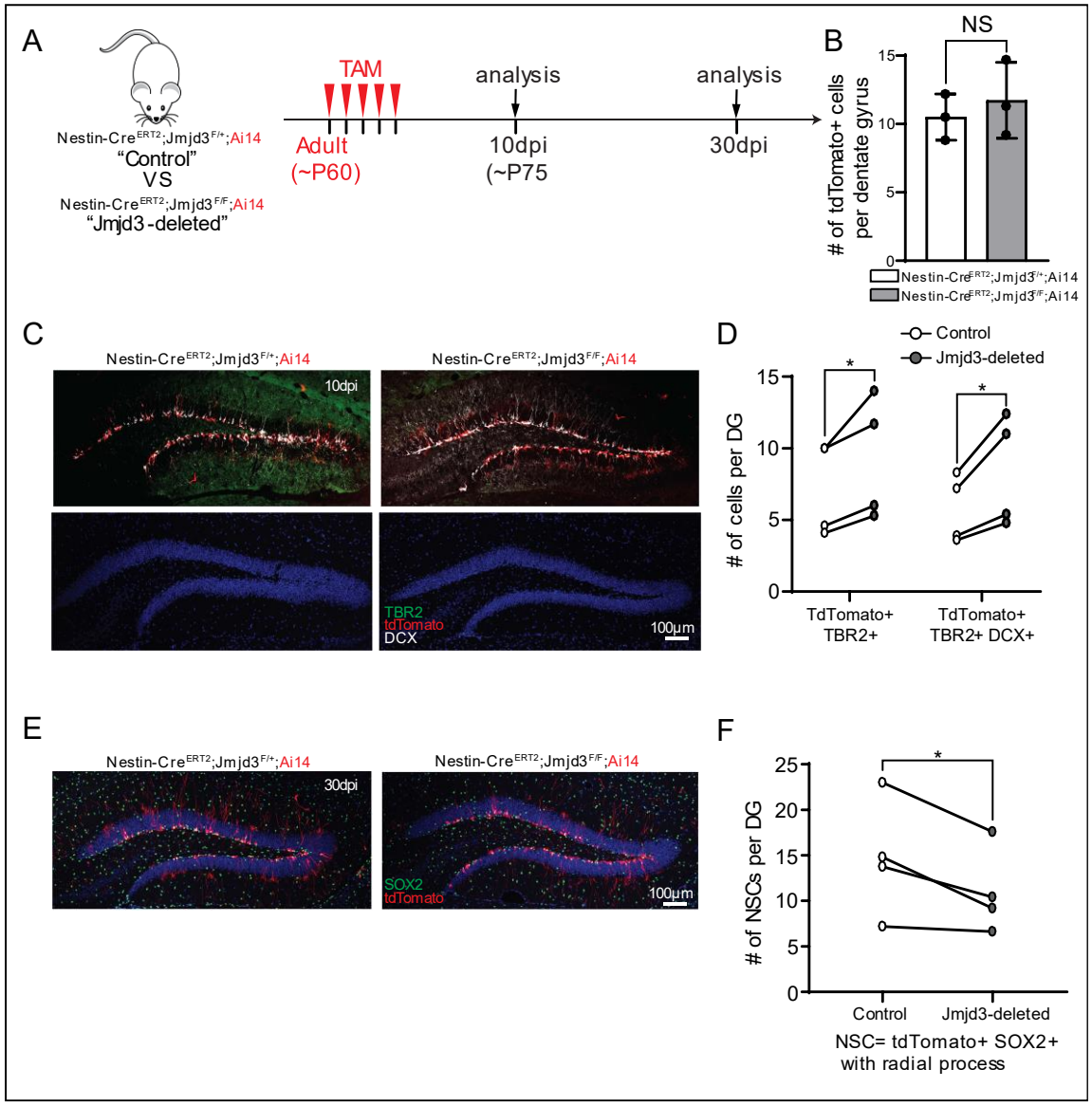

Figure 3.4 Jmjd3 is required for maintenance of adult SGZ NSCs
* = p < 0.05, NS = not significant
A, Schematic of experimental design. **B,** Quantification of number of tdTomato+ cells at the end of TAM administration comparing Nestin-Cre^{ERT2};*Jmjd3$^{F/F}$*;Ai14 to control. Two-tailed unpaired t test. **C,** IHC for tdTomato (red), TBR2 (green), DCX (white), and DAPI in coronal sections of Nestin-Cre^{ERT2};*Jmjd3$^{F/F}$*;Ai14 to control 10dpi. **D,** Quantification of (C), two-tailed paired t test. **E,** IHC for tdTomato (red), SOX2 (green), and DAPI (blue) in coronal sections of Nestin-Cre^{ERT2};*Jmjd3$^{F/F}$*;Ai14 to control 30dpi. F, Quantification of (E), two-tailed paired t test.

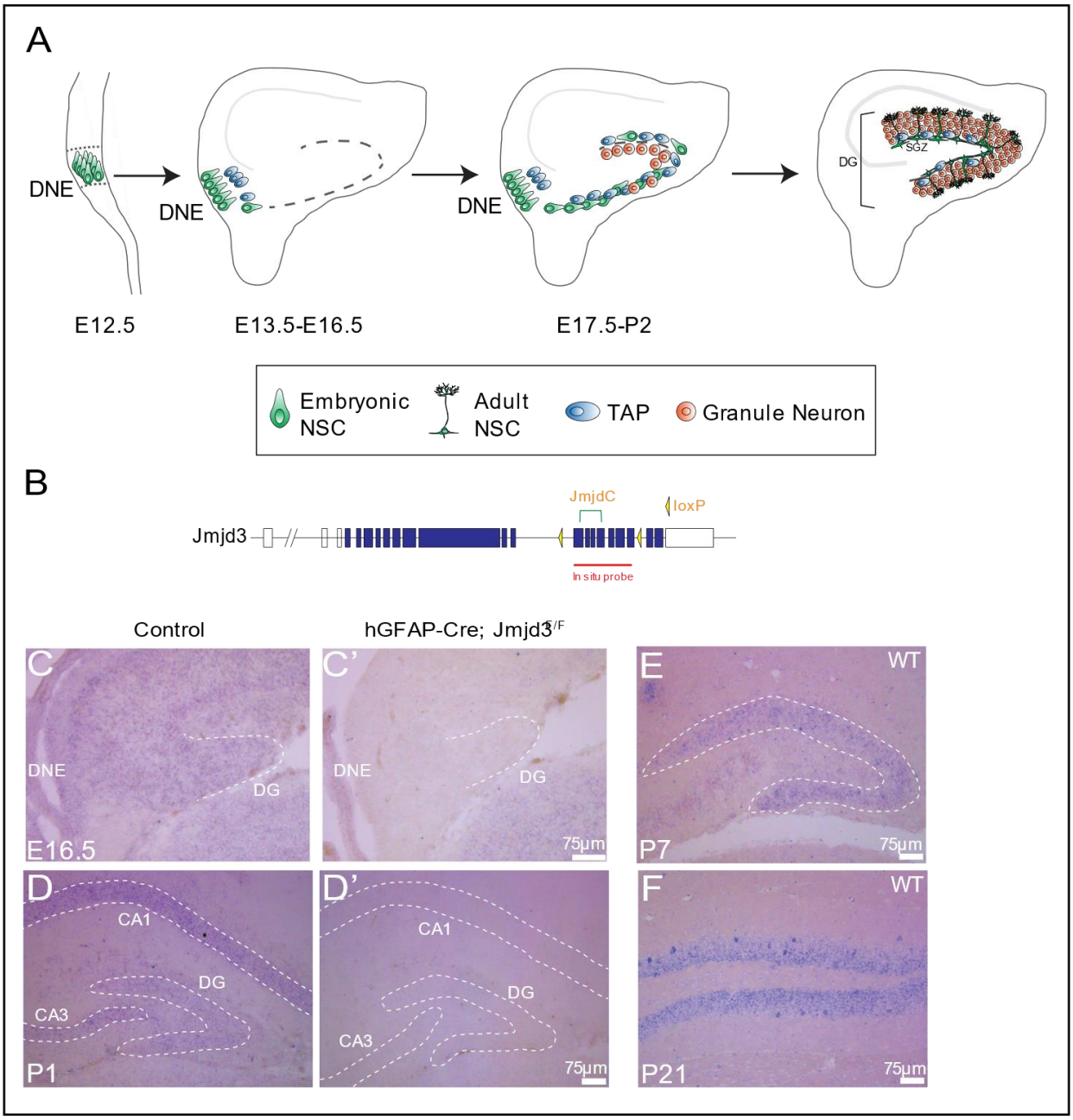

Figure 3.S1 *Jmjd3* is expressed in the developing hippocampal dentate gyrus (DG)

A, Schematic illustration of DG development. **B,** Schematic illustration of *Jmjd3* floxed allele and position of *in situ* hybridization (ISH) probe. **C-D',** ISH for Jmjd3 in coronal DG sections of control (C-D) and Jmjd3-deleted mice (C'-D') at E16.5 (C-C') and P1 (D-D'). **E-F,** ISH for Jmjd3 in coronal DG sections of control at P7 (E) and P21 (F). DNE: Dentate neuroepithelium SGZ: Subgranular zone

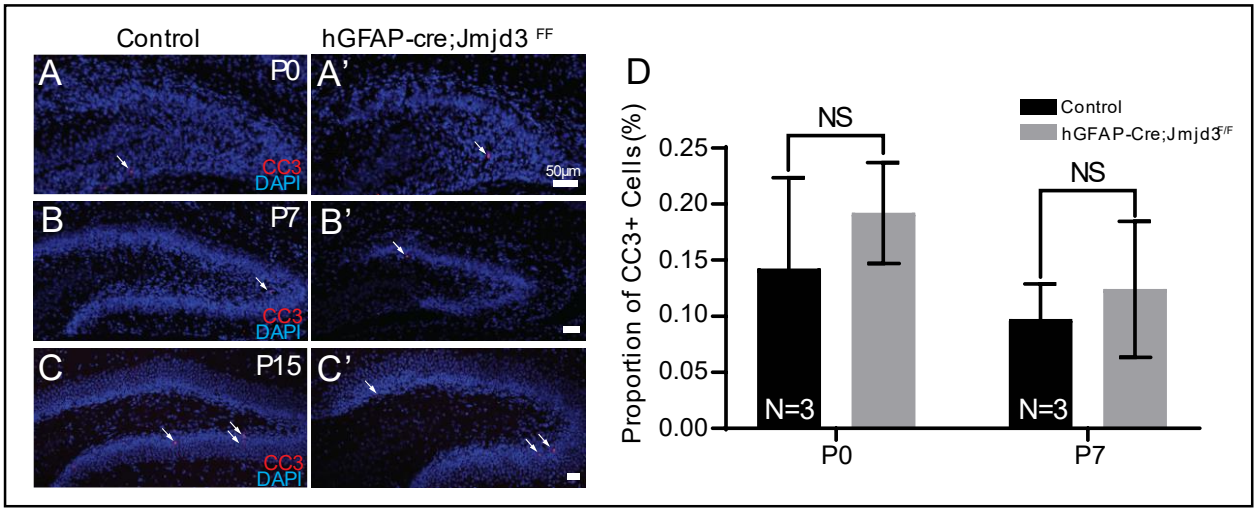

Figure 3.S2 Increased cell death is not observed in *hGFAP-Cre;Jmjd3$^{F/F}$* mice
A-C', IHC for cleaved caspase 3 (CC3) (red) and DAPI (blue) in coronal DG sections of P0 to P15 control (A-C) and *hGFAP-Cre;Jmjd3$^{F/F}$* mice (A'-C'). **D,** Quantification of (C) two-tailed unpaired t test, NS = not significant.

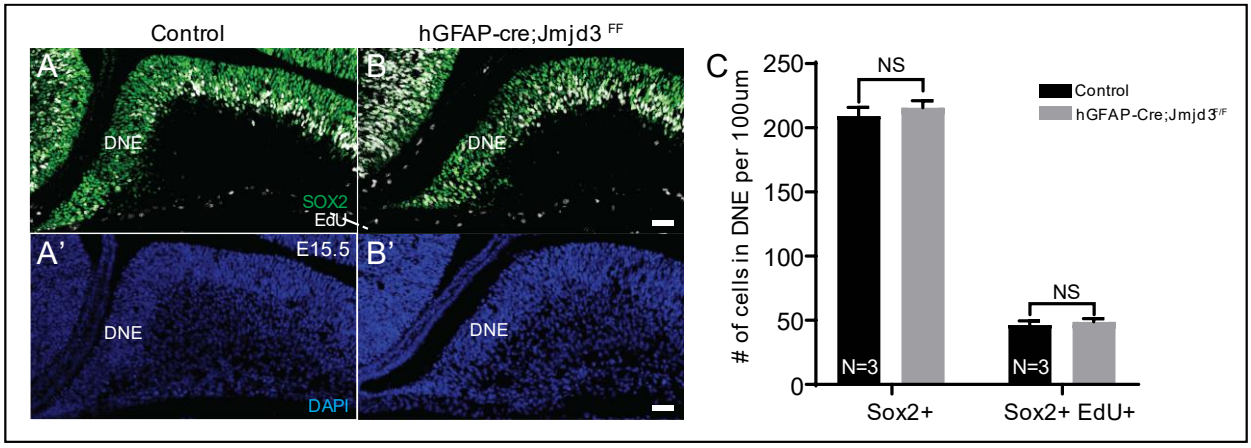

Figure 3.S3 Similar number of SGZ NSC precursor cells at E15.5 between Jmjd3-deleted and control brain

A-B', IHC for SOX2 (green), EdU (white) and DAPI (blue) in coronal DG sections of E15.5 control (A-A') and *hGFAP-Cre;Jmjd3$^{F/F}$* mice (B-B'). **C,** Quantification of (A) two-tailed unpaired t test. NS = not significant

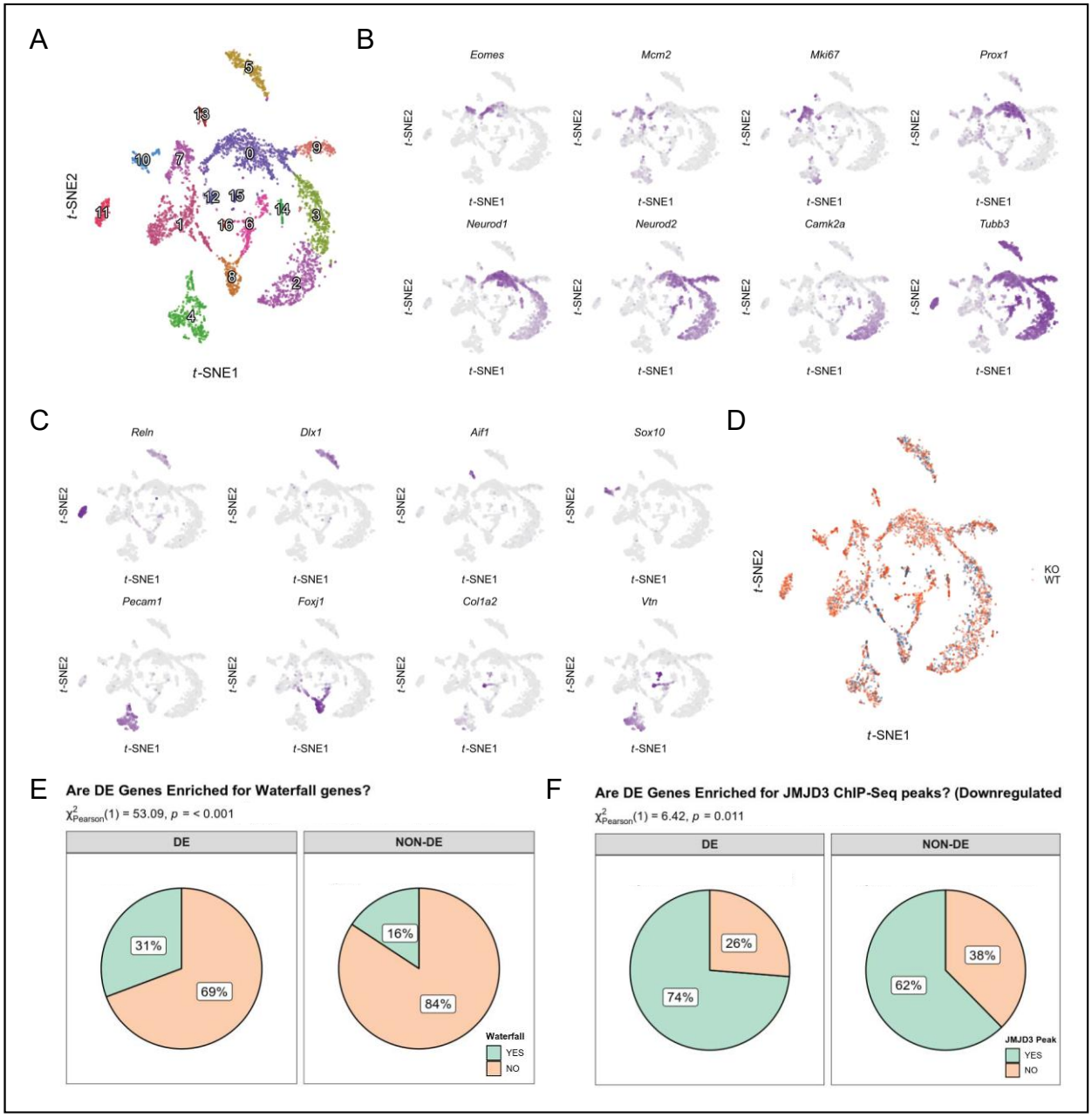

Figure 3.S4 Single-cell RNA sequencing resolves the tissue heterogeneity in developing DG

A, unlabeled t-SNE plot of DG cells from hGFAP-Cre;*Jmjd3*$^{F/F}$ and control mice. **B,** t-SNE plot of well-known marker expression for each cell type. **C,** t-SNE plot of known NSC marker. **D,** t-SNE plot of DG cells based on the genotype of mice. **E,** Correlation analysis between enrichment of differentially expressed genes with genes from previously published paper (Shin et al., 2015). **F,** Correlation analysis between differentially expressed genes and JMJD3 ChIP-seq data from NSCs derived from E12.5 mouse cortex (Fueyo et al., 2018).

Experimental Procedures

Mus musculus

Jmjd3 $^{F/F}$ mice which contain *Jmjd3* alleles with loxP sites flanking the JmjC catalytic domain were maintained and genotyped as described (Iwamori et al., 2013; Park et al., 2014). Mice of both sexes were included in all experiments, and were analyzed at multiple time point between E15.5 and P60 as described in the text and figure legends for each corresponding experiment. All samples were analyzed relative to littermate control mice. Experiments were performed in accordance to protocols approved by Institutional Animal Care and Use Committee at UCSF. For the purpose of the adult fate-tracing, >P60 mice received 5mg of TAM (Sigma) dissolved in 100% corn oil (Sigma) by oral gavage per 30 grams of the body weight once a day for 5 consecutive days.

hGFAP-Cre: Tg(GFAP-Cre)25Mes/J, described in (Zhou et al., 2001).

Nestin-Cre^{ERT2}: Tg(Nes-cre/ERT2)KEisc/J, described in (Lagace et al., 2007).

Ai14: Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$, described in (Madisen et al., 2010).

BrdU administration

Mice were injected 5-bromo-2'-deoxyuridine (BrdU, Millipore Sigma) reconstituted in sterile PBS (10mg/ml) intraperitoneally at a dose of 50mg of BrdU per kg in mouse weight

Immunohistochemistry

Brains were fixed by intracardiac perfusion (Park et al., 2014) and sectioned on a cryostat (Leica) at 14 μm thickness or a vibratome (Leica) at 40 μm thickness. After blocking with 10% normal goat serum, 0.3% Triton-X 100, 1% bovine serum albumin,

and 0.3M glycine in PBS for 1hr at room temperature, primary antibodies were incubated at 4 °C overnight. For JMJD3 staining, rabbit anti-JMJD3 antibodies (Abgent) were affinity purified using EpiMAX Affinity Purification kit (Abcam) and epitope retrieval was performed with 2N HCl. Fluorescence signal was amplified using TSA Plus fluorescence kit (PerkinElmer). The following primary antibodies were used in this study: rabbit anti-JMJD3 (epitope purified, 1:5, Abgent), rat anti-GFAP (1:500, Invitrogen), guinea pig anti-Doublecortin (1:1000, Millipore), rat anti-BrdU (1:500, Abcam), mouse anti-NeuN (1:500, Chemicon), mouse anti-NESTIN (1:500, Millipore), rabbit anti-Cleaved Caspase 3 (1:250, Covance), rabbit anti-Ki67 (1:500, Abcam), rabbit anti-NeuroD2 (1:300, Abcam), rat anti-Ctip2 (1:300, Abcam), mouse anti-Calbindin1 (1:300, Swant), goat anti-SOX2 (1:300, Santa Cruz) , rabbit anti-PROX1 (1:300, Covance). Goat or Donkey Alexa-Fluor secondary antibodies (Invitrogen) were used, and nuclei were counterstained with DAPI (Sigma).

Single-cell RNA-seq Analysis

DG were micro-dissected from P2 hGFAP-Cre*;Jmjd3*$^{F/F}$ mice and littermate controls (2 animals per condition) and dissociated into single cells using the Worthington Papain Dissociation system following all manufacturer protocol steps including ovomucoid gradient. Single-cell libraries were generated using the 10x Genomics Chromium Single Cell 3' Assay with a targeted cell recovery of 5000 (2500 cells per condition). Libraries were sequenced to a mean depth of approximately 55,000 reads/cell and processed through cellranger 3.0.2 (10x Genomics) with a raw recovery of 5847 cells (3555 WT and 2292 KO). All subsequent processing and analyses were performed using the Seurat v3 R package. Quality filtering was performed on the basis

mitochondrial and ribosomal content before log normalization and scaling. WT and KO datasets were then integrated using canonical correlation analysis and the integrated dataset was used for dimensionality reduction by principal components analysis (PCA) and *t*-distributed stochastic neighbor embedding (*t*-SNE). Cluster analysis on the integrated was conducted using the Louvain algorithm on the shared nearest neighbor network, with resolution set to 0.4. Major cell types were assigned to clusters using canonical markers from the experimental literature as well as other published scRNA-seq datasets of the rodent hippocampus. Differential expression testing was performed between WT and KO cells within pertinent clusters using the likelihood-ratio test for single-cell gene expression ("bimod" setting) and p-values were adjusted using the Bonferroni correction. Enrichment analysis of DE genes for other datasets (e.g. JMJD3 ChIP-Seq, DG NSC waterfall genes) were conducted using the Fisher Exact test.

Microscopic Analysis

For *in vivo* DG cell quantification, I used at least 3 animals per group. Confocal images were obtained with a Leica TCS SP5X with 20X objective; from each animal, at least three separate coronal sections including both hemispheres were analyzed using ImageJ. Statistical tests of significance were performed using paired t-Test in GraphPad Prism.

Human Fetal Tissue

Fetal hippocampal tissue (GW16) was collected from elective pregnancy termination specimens at San Francisco General Hospital, usually within 2 hours of the procedures. Research protocols were approved by the Committee on Human Research (institutional review board) at University of California, San Francisco.

In Situ Hybridization (ISH)

ISH on brain tissue was performed as previously described (Wallace and Raff, 1999) with DIG-labeled RNA probe designed against JmjC domain of *Jmjd3*. ISH images were obtained using a DMI4000B microscope (Leica).

Chapter 4: Conclusions and Future Directions

While most embryonic NSCs stop proliferating at birth, a subset of them transitions to establish the adult NSC pool in restricted regions where they last through the life of the animal (Kriegstein and Alvarez-Buylla, 2009). V-SVZ NSCs give rise to neuroblasts, which then migrate to the olfactory bulb (OB) where they differentiate to become inhibitory interneurons (Altman 1969; Lois and Alvarez-Buylla, 1994). In contrast, SGZ NSCs in the hippocampus give rise to excitatory granule neurons (Kitabatake et al., 2007). At the cellular level, NSCs produce new neurons that are able to become integrated into pre-existing neural circuits (Belluzzi et al., 2003; Carleton et al., 2003). But a key question is whether adult NSCs have relevance to brain and cognitive function.

Accumulating studies suggest that adult neurogenesis may be associated with corresponding brain functions in each adult NSC niche. For V-SVZ-OB neurogenesis, mice exposed to an odor-enriched environment exhibited an increased neuronal production in the OB compared to controls (Rochefort et al., 2002). Interestingly, this increased neuronal production was not observed in the hippocampus suggesting the specificity to olfactory memory not spatial memory. In contrast, mice that performed various spatial learning tasks exhibited an increase in number of newly born neurons in the dentate gyrus (Kitabatake et al., 2007). While these studies may address potential roles of NSCs, it is important to recognize that there are also other studies showing either no effect or even a decrease in neuronal production (Pham et al., 2005; Merrill et al., 2003). Therefore, further *in vivo* studies are required to assess the correlation between adult neurogenesis its role in cognition and memory.

With the availability of various transgenic mouse lines, there have been advancements in understanding the exact roles that adult NSCs play for cognition. When Diphtheria toxin A (DTA) was targeted to selectively kill Nestin+ NSCs in the adult brain, mice exhibited defects only in hippocampal-mediated behaviors and not olfactory-mediated behaviors (Imayoshi et al., 2008). Even though the DTA killed adult NSCs in both SVZ and SGZ, only spatial memory was impaired despite huge reduction of OB neurogenesis, suggesting that the functional relevance of adult NSCs may be context-dependent. In addition, impaired hippocampal neurogenesis has been associated with depression, neuro-inflammation, epilepsy, and other numerous neurological disorders (Dranovsky and Hen, 2006; Parent et al., 2006; Anacker and Hen, 2017). Thus, understanding requirements for long-term NSC maintenance may provide critical insights into how NSCs contribute to essential brain functions coupled with how their dysfunctions may lead to neurological disorders.

NSC's self-renewal and differentiation are modulated by both cell-intrinsic and -extrinsic factors during development and adulthood. Cell-intrinsic regulators include signaling pathways (e.g. Wnt, Notch, Bmp, Shh, Fgf, and others) or transcription factors expressed by NSCs that directly control NSCs' self-renewal and differentiation (Patten et al., 2006; Gaiano and Fishell, 2000; Panchision et al., 2010; Imayoshi et al., 2010; Arredondo et al., 2019). Extrinsic factors consist of extracellular signaling are produced by other cell types in the NSC (Urban et al., 2014). Insulin or insulin-like growth factors (Igfs) signaling from cerebrospinal fluid (CSF) has been implicated in NSC regulation (Woods et al., 2003). Insulin signaling may interact with proneural transcription factor Ascl1 to promote activation of the NSC pool. Neurotransmitters such as GABA (γ-

aminobutyric acid) and acetylcholine are other examples of extracellular signaling essential for NSC functions. GABA, which is released from inhibitory interneurons, can promote neuronal differentiation and inhibit proliferation in embryonic NSCs. In adult NSCs, deletion of GABA receptors ($GABA_AR$ and $GABA_BR$) may increase embryonic NSC proliferation (Giachino et al., 2014a; Song et al., 2012). Conversely, acetylcholine is an excitatory neurotransmitter that has been shown to have opposing NSC regulatory function (Berg et al., 2013).

This dissertation demonstrates that a chromatin regulator and a long noncoding RNA are both cell-intrinsic regulators for long-term maintenance of NSCs. Though our current understanding of regulators of NSC function during development mostly revolve around signaling pathways or transcription factors, it is now clear that other classes of genes also play critical roles (Urban et al., 2014). Mutations of chromatin regulators have been associated with human neurodevelopmental and psychiatric disorders (De Rubeis et al., 2014; Iossifov et al., 2014; Sanders et al., 2015). Depletion of adult NSCs exhibit cognitive deficits in mice (Imayoshi et al., 2008). Therefore, gaining better understanding of how NSCs are modulated during development and into adulthood will provide critical insights into the mechanisms underlying neurodevelopmental disorders.

Using multiple genetic approaches, I have shown that the lncRNA-*Pnky* regulates NSC function in the neocortex. Loss of Pnky leads to increased neuronal differentiation, despite its close proximity to *Pou3f2* gene. *Pnky* expression from BAC^{Pnky} is sufficient to rescue the embryonic and postnatal phenotypes resulting from *Pnky* deletion, suggesting that it functions in *trans*. Based on impaired NSC functions during cortical development observed in $Pnky^{\Delta/\Delta}$ mice, I hypothesized that $Pnky^{\Delta/\Delta}$ mice may exhibit

behavioral phenotypes due to the well-known association between the cortex and cognitive function (Molyneaux et al., 2007). Indeed, adult *Pnky*$^{\Delta/\Delta}$ mice (~3 months old) exhibited an impairment in acoustic startle threshold, pre-pulse inhibition, and cued fear conditioning (**Table 2.1**). Although this behavior phenotype was sex-dependent, these data suggest that *Pnky*-deletion may impair cognitive functions. To my knowledge, this is one of the very first studies suggesting roles for a lncRNA in cognition. It would be interesting then to investigate whether BAC^{Pnky} can also rescue this behavior phenotype, to more conclusively demonstrate that *Pnky* directly influences cognition through its effects on NSC functions.

Pnky has been shown to be conserved in the human with similar expression pattern. Similar to mice, *PNKY* is also enriched in the VZ of developing human cortex and human cerebral organoids (Ramos and Andersen et al., 2015). Given the conservation of expression pattern and sequence between mouse *Pnky* and human *PNKY*, it would be interesting to determine whether this lncRNA plays a role in human NSC function and brain development. In addition, aside from the VZ of the developing human and mouse cortex, *PNKY* is expressed in the cortical plate where projection neurons reside (Ramos and Andersen et al., 2015). Given that lncRNAs are highly cell-type specific, understanding the function of *PNKY* in postmitotic neurons would further broaden the knowledge about the role that lncRNAs play in cell biology.

My studies also show that the chromatin regulator *Jmjd3* is required for the establishment and long-term function of the NSC niche in the hippocampus. Loss of *Jmjd3* leads to a defective stem cell maintenance gene expression signature and an accompanying impairment in maintenance of adult NSCs. Similar to previous studies

using DTA to selectively kills NSCs, NSC depletion from *Jmjd3*-deletion result in hippocampal-dependent cognitive function (data not shown). Essential roles of chromatin regulators in NSC maintenance may explain why chromatin regulators are often mutated in human neurodevelopmental disorders (Najmabadi et al., 2011; Rubenstein, 2011).

A key next step would involve identifying the molecular mechanism through which *Jmjd3* acts. *Jmjd3* is involved in various cell biological processes, including differentiation, proliferation, lineage specification, reprogramming, and senescence in multiple organs, suggesting that its role may be context dependent (Burchfield et al., 2015). Even within the brain, its function may vary in different regions and cell types. A previous study of *Jmjd3* in V-SVZ NSCs show that JMJD3 and its demethylase activity is required to activate neurogenic gene expression via interactions at promoters and enhancers (Park et al., 2014). Unlike the loss of SGZ NSCs, deletion of *Jmjd3* in the V-SVZ causes an accumulation of V-SVZ NSCs. This may be due to interactions with different cell type-specific binding partners or employment of different mechanisms of action.

A recent study in which 30,416 cells from the human hippocampus were sequenced between gestational weeks 16-27 revealed that JMJD3 is also expressed in the developing human hippocampus (Zhong et al., 2020). It would thus also be interesting to determine whether the role of *JMJD3* in human NSC function is similar to that observed in the mouse.

Further investigating the potential role of *Jmjd3* in cellular metabolism would also be of great interest. JMJD3 uses α-KG as one of the co-factors for its demethylase

activity. α-KG, one of the intermediates of the TCA cycle, is converted to succinate (Klose et al., 2006). The balance between the concentration of α-KG and succinate has been previously associated with stem cell differentiation, where α-KG has been shown to accelerate the initial differentiation of primed human stem cells (Carey et al., 2015; TeSlaa et al., 2016). Interestingly, accelerated differentiation was reversed when succinate was introduced to balance the concentration gradient. Therefore, I speculate that *Jmjd3* deletion may lead to increased [α-KG] and a decreased in [succinate], which may explain the disruption of metabolic genes in *Jmjd3*-deleted NSCs.

In this dissertation, I have shown that JMJD3 and lncRNA Pnky regulates NSC function. Given that normal stem cells and cancer stem cells utilize similar molecular mechanisms of self-renewal, I speculate that similar regulatory principles may be relevant for cancer biology. Glioblastoma (GBM) is one of most aggressive brain tumors where self-renewing GBM stem cells have been associated with high tumorigenicity and poor survival rate (Lathia et al., 2015). A transcription factor, STAT3, is implicated as a key modulator for both NSCs and GBM stem cells (Sherry-Lynes et al., 2017; Wang et al., 2015). Previous studies show that *Jmjd3* may be a direct target of STAT3 regulating self-renewal of GBM stem cells responsible for long-term maintenance (Sheey-Lynes et al., 2017). *Jmjd3* has also been implicated in regulating other types of cancer stem cells (e.g. prostate, brain, gastric, breast, skin, and others) (Yin et al., 2019). Since chromatin regulators can exhibit cell type-specific expression and function, they may be ideal therapeutic candidates for cancer. In addition, many lncRNAs are differentially expressed in various types of cancers and tumors (Andersen and Lim, 2018). Recent data suggests that depletion of lncRNAs using an ASO can selectivity kill tumor cells,

indicating potential to serve as a therapeutic approach (Liu and Malatesta et al., 2020). Therefore, deeper insights into how chromatin regulators and lncRNAs function may not only contribute to our basic understanding of molecular biology and development, but also may serve as a foundation for future therapeutics for various types of diseases.

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