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Chromatin-modifying factor Mll1 maintains neural stem cell regional identity in the postnatal mouse brain

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Chromatin-modifying factor Mll1 maintains neural stem cell regional identity in the postnatal mouse brain

by

Ryan N Delgado

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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of the

For my family

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Contributions to presented work

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

Chapter 3 is a manuscript prepared for submission with the following authors: Ryan N. Delgado and Daniel A. Lim. I designed, performed, and analyzed the experiments. Daniel Lim supervised research. Together, we wrote the manuscript.

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Chromatin-modifying factor Mll1 maintains neural stem cell regional identity in the postnatal mouse brain

By
Ryan N. Delgado

Abstract

The adult ventricular-subventricular zone (V-SVZ) contains neural stem cells (NSCs) that produce neurons throughout life. NSCs in the V-SVZ are regionally heterogeneous such that those located ventrally generate different subtypes of neurons than those located dorsally. Whether epigenetic mechanisms underlie the maintenance of the regional identity of NSCs is not known. Transcriptional analysis of dorsal and ventral V-SVZ NSCs revealed an enrichment of homeotic transcription factors consistent with the embryonic origins of each region. Specifically, ventral V-SVZ NSCs were enriched for *Nkx2.1*, a transcription factor required to specify the ventral telencephalon during embryogenesis. Using *Nkx2.1*-CreER fate tracing, we found that *Nkx2.1*+ V-SVZ NSCs generated OB interneurons in adulthood. Embryonic fate-labeling revealed that early embryonic *Nkx2.1*+ cells are direct precursors to the NKX2.1+ NSCs of the postnatal V-SVZ. While sonic hedgehog (Shh) signaling is required to induce and maintain *Nkx2.1* expression early in neural development, inhibition of Shh signaling by cyclopamine and vismodegib did not reduce *Nkx2.1* expression in postnatal V-SVZ NSCs, suggesting that *Nkx2.1* expression in the V-SVZ was being maintained epigenetically. Chromatin analysis of dorsal and ventral V-SVZ NSCs revealed *Mixed-lineage leukemia 1* (MLL1) protein, a trithorax group member and H3K4 methytransferase, was enriched at the *Nkx2.1* locus in ventral, but not dorsal V-SVZ NSCs. Conditional deletion of *Mll1* in ventral V-SVZ NSC cultures resulted in loss of *Nkx2.1* expression, but did not impair Shh pathway

signaling, suggesting that *Mll1* is required for the maintenance of Shh-independent Nkx2.1 expression. These data thus illustrate how the positional information provided by extrinsic cellular signals such as morphogens becomes epigenetically encoded into regionally distinct cell types. More generally, these results help explain how complex developmental information is properly maintained despite rapid tissue growth and/or dynamic changes in morphogen gradients.

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CHAPTER 1: INTRODUCTION

Section 1: Positional Identity

Cell fate determination in the preimplantation embryo

The developmental fate of precursor cells is heavily dependent on their position within the developing embryo. Cells first acquire different positions relative to each other when blastomeres undergo compaction to form the morula, causing some cells to be positioned more superficially than others (Johnson and McConnell, 2004). In mice this happens at the 8-cell stage (Glasser et al., 1981),and in humans at the 16-cell stage (Edwards et al., 1981; Trounson et al., 1982). Fate-tracing studies of both intact and dissociated preimplantation embryos demonstrate that blastomeres in the 2-, 4-, and 8-cell mouse embryo are totipotent and generate both trophectoderm and the inner cell mass lineages at high frequency (Balakier and Pedersen, 1982; Pedersen et al., 1986; Tarkowski and Wroblewska, 1967). The developmental potential of blastomeres is highly plastic at this point (Tarkowski and Wroblewska, 1967) and can be altered by manipulating their relative position (Hillman et al., 1972). However, over the next two cell divisions blastomeres become increasingly restricted in cell fate and by the end of the 32-cell stage the outer-most cells are largely committed to generating the trophoectoderm while the cells located in the center of the embryo generate the inner cell mass (Dyce et al., 1987; Pedersen et al., 1986). Thus, cells obtain unique “positional identities” that guide their developmental potential very early in development. While the mechanisms by which positional identity are generated and subsequently interpreted in the preimplantation embryo are still not fully understood (Cockburn and Rossant, 2010; Watanabe et al., 2014), positional identity clearly plays an important role in the very first lineage restriction of the developing embryo.

Establishment of positional identity

As development progresses, the positional identity of embryonic precursors becomes increasingly complex as the major anatomical axes emerge and the body plan is elaborated. Classic developmental studies of *Drosophila* reveal that early embryonic precursors are "patterned" into distinct spatial populations through the actions of regionally expressed transcription factors (St Johnston and Nusslein-Volhard, 1992). This early body of work provided key insights into how positional identity is both established and maintained during development.

Shortly after the onset of gastrulation, transcription factors encoded by gap and pair-rule genes, such as *Krüppel* and *paired,* pattern the anterior-posterior axis into 14 repeating units known as parasegments (Akam, 1987). Evidence of segmentation can be observed as early as 5 hours post fertilization when grooves appear between each parasegment (Martinez-Arias and Lawrence, 1985). In addition to establishing a segmented body plan, gap and pair-rule genes induce the expression of a second class of patterning genes, the homeotic (*Hox*) transcription factors (McGinnis and Krumlauf, 1992; White and Lehmann, 1986). The combinatorial expression of *Hox* genes guides the developmental fate of each parasegment (Maeda and Karch, 2006). *Hox* genes are highly conserved across evolution and are arranged linearly in the genome in approximately the pattern in which they are expressed across the embryo (Pearson et al., 2005). Together, these two classes of genes control the positional identity of the developing embryo.

The phenotypes exhibited by gap and pair-rule mutants are significantly different from those seen in *Hox* mutants, indicating that they regulate two distinct developmental processes. In the absence of wildtype gap and pair-rule gene function, the developing embryo fails to generate the appropriate number of parasegments, indicating that these genes are required for pattern formation (Nusslein-Volhard and Wieschaus, 1980). In contrast, loss-of-function *Hox* gene mutations do not result in altered segment number, but rather in the altered developmental

fate of segments (McGinnis and Krumlauf, 1992). For example, homeotic gene *Ubx* is required to for the development of an appendage known as a haltere in the third thoracic segment. *Ubx* mutants fail to generate halteres, but instead develop an additional set of wings, a feature normally developed in the adjacent parasegment (Lawrence and Morata, 1983; Ouweneel and Vanderme.Jm, 1973). This shift in fate is known as a "homeotic shift" and demonstrates that *Hox* gene expression is required to specify the developmental fate, and thus positional identity, of each segment.

Maintenance of positional identity

Shortly following the establishment of *Hox* gene expression, gap and pair-rule genes are downregulated, suggesting that the *Hox* gene expression is maintained independent of the signals that drove its initial expression (Akam, 1987; Nusslein-Volhard and Wieschaus, 1980). Furthermore, once specified, the developmental fate of precursors in a particular parasegment is remarkably robust. Pioneering studies performed by Walter Gehring and Ernst Hadorn found that imaginal discs from specific parasegments could be removed and cultured for extended period of time (up to 5 years) in the abdominal cavity of an adult fly where they continue to proliferate in an undifferentiated state (reviewed in (McClure and Schubiger, 2007; Worley et al., 2012)). Once allowed to develop, they found that the imaginal discs retained the developmental fate of the parasegment from which they were derived. These results demonstrated that positional identity could be maintained stably and heritably, independent of the signal that was responsible for its induction.

Significant insights into how positional identity could be maintained in a cell-autonomous manner came from studies of *Polycomb* and *trithorax* group (*PcG* and *trxG*) mutants in *Drosophila* (Ringrose and Paro, 2004). While *PcG* and *trxG* mutants are able to initiate proper *Hox* gene patterning, they are unable to maintain it (Ernst et al., 2004; Struhl and Akam, 1985;

Yu et al., 1998). *PcG* mutants were identified first in the 1940s; screens performed to identify mutations that rescued *PcG* mutants later led to the discovery of *trxG* genes (Ingham and Whittle, 1980; Ingham, 1998; Kennison and Tamkun, 1988; Ringrose and Paro, 2004). While PcG genes act as transcriptional repressors, trxG genes function as transcription activators (Schuettengruber et al., 2007). Together, *PcG* and *trxG* genes function antagonistically to maintain the appropriate expression of *Hox* genes long after the expression of gap and pair-rule gene has subsided (Steffen and Ringrose, 2014). In the absence of proper *PcG* and *trxG* function[1], *Hox* genes become misexpressed, resulting in homeotic shifts (Lewis, 1978).

The roles of *PcG* and *trxG* in maintaining regional gene expression are conserved across evolution. The prototypical *trxG* gene *trithorax (trx)* and its mammalian homolog *Mixed-lineage leukemia 1 (Mll1)* are both required to positively maintain *Hox* gene expression (Ingham and Whittle, 1980; Yu et al., 1998). *Mll1*-null mice die embryonically (~E10.5), but *Mll1*-heterozygotes are carried to term and exhibit overt defects in their axial skeleton (Yu et al., 1995). Consistent with phenotypes observed in *Drosophila*, *Hox* gene expression is established normally within *Mll1* mutants, however it becomes lost shortly afterward (Yu et al., 1998). Homeotic transformations are also observed in mice null for *Bmi1*, a *PcG* gene (van der Lugt et al., 1994). Thus, *PcG* and *trxG* genes are key components of a "cellular memory system" required to maintain *Hox* gene expression throughout development.

Mechanisms that maintain gene expression in a stable and heritable manner in the absence of the initiating signal and without changes in the DNA sequence are said to be "epigenetic".[2] *PcG* and *trxG* members are important regulators of epigenetic memory and serve

[1] As is common in *Drosophila* nomenclature, *PcG* and *trxG* genes are named for the phenotypes that arise from their mutation. *PcG* mutants *Polycomb* (*Pc*) and *extra sex combs* (*esc*) exhibit the aberrant formation of sex combs, a feature usually confined to the first leg, on their second and third leg (Ringrose and Paro, 2004). *trxG* genes derive their name from the *trithorax* (*trx*) mutant which displays developmental abnormalities in all three thoracic segments (Ingham, 1998).

[2] For a discussion on the misuse of the term "epigenetic", see: (Ptashne, 2007, 2013).

to enforce the memory of active and repressed transcriptional states throughout development. MLL1 contains a highly-conserved SET domain and can function as a histone methyltransferase for lysine 4 in histone H3 (H3K4) (Gu et al., 1992; Mazo et al., 1990; Rozovskaia et al., 2000; Schuettengruber et al., 2011). However, MLL1's methyltransferase activity does not seem to entirely account for its role as an epigenetic maintenance factor. *Mll1* SET domain mutants are born at expected mendelian frequencies and display relatively minor defects in axial patterning compared to mice heterozygous for a null *Mll1* allele (Terranova et al., 2006; Yu et al., 1995). *Mll1* is unique in that, unlike many other *PcG* histone methyltransferases, it remains associated with condensed mitotic chromosomes, leading to the idea that it functions as a "bookmark" capable of rapidly re-establishing gene expression patterns following mitosis (Blobel et al., 2009; Schuettengruber et al., 2011). Future studies will hopefully uncover the exact mechanisms underlying *Mll1*-mediated inheritance of transcription programs.

Section 2: Neural precursor diversity in the developing brain

Diversity within the CNS

The central nervous system (CNS) contains a diverse array of neuronal and glial cell types required for its proper function. Precursors in the embryonic brain are regionally heterogeneous and generate different types of neurons[3] (Hebert and Fishell, 2008; Jessell, 2000). Like the developing *Drosophila* embryo, embryonic neural precursors throughout the murine CNS are patterned into distinct domains by regional transcription factor expression (Dasen and Jessell, 2009; Flames et al., 2007; Shimamura et al., 1995; Tumpel et al., 2009). The establishment and maintenance of these domains are important aspects of proper brain development.

The brain arises from a population of pseudostratified epithelial cells that derive from the ectoderm (Price, 2011). By embryonic day 9-10 (E9-10), neuroepithelial cells develop into neural precursors known as radial glia cells (RGCs) (Kriegstein and Alvarez-Buylla, 2009). RGC cell bodies reside in the ventricular zone (VZ) and extend processes that contact both the ventricular and pial surfaces. In addition to their unique radial morphology, RGCs can be identified by their expression of the intermediate filament protein encoded by *Nestin*, which is also recognized by the RC2 antibody (Misson et al., 1988; Park et al., 2009), as well as brain lipid-binding protein (BLBP) (Feng and Heintz, 1995). BLBP is expressed by RGCs throughout the developing CNS (Feng et al., 1994) and fate-mapping experiments performed with BLBP-Cre mice that express Cre under the regulation of the BLBP promoter label neurons throughout the CNS, indicating that RGCs function as neural precursors throughout the developing CNS (Anthony et al., 2004). Similar fate-mapping experiments performed using Cre-lines under the regulation of regionally expressed transcription factors label restricted subpopulations of neurons (Taniguchi et al., 2011; Xu et al., 2008; Young et al., 2007). These data support the notion that neural precursors in the mammalian brain are patterned into distinct regional populations by the actions of fate-specifying transcription factors.

Shh signaling and *Nkx2.1* expression

The establishment of regional transcription factor patterns in the developing brain requires the inductive actions of morphogens such as Sonic hedgehog (Shh) (Hebert and Fishell, 2008; Monuki and Walsh, 2001; Wilson and Rubenstein, 2000). The hedgehog signaling pathway is conserved across evolution (Ingham and Placzek, 2006) and plays an important role in the specification of ventral precursors throughout the developing nervous system (Chiang et

[3] Astrocyte heterogeneity is important for proper brain function and is itself a rapidly growing field (Molofsky et al., 2012; Zhang and Barres, 2010), however for the sake of brevity, I am choosing to focus on neuronal diversity.

al., 1996; Echelard et al., 1993; Ericson et al., 1995). *Shh* encodes a soluble ligand that signals through the obligate transmembrane receptors *Ptch1* and *Smo* (Alcedo et al., 1996; Stone et al., 1996), resulting in the dose-dependent induction of transcription factor expression across the dorsoventral axis of the developing brain (Balaskas et al., 2012; Dessaud et al., 2007). The inductive actions of Shh singling are best conceptualized in the spinal cord where Shh signaling is required to specify the fate of ventral precursors (Briscoe et al., 1999; Ericson et al., 1997; Novitch et al., 2001). Shh signaling exhibits both inductive and repressive effects on gene expression, ensuring that transcription factors that encode ventral fates are expressed ventrally while transcription factors that promote dorsal fates are repressed (Fuccillo et al., 2006; Jessell, 2000). Thus, Shh signaling induces the formation of spatially distinct precursors domains in the ventral spinal cord.

Like the spinal cord, the precursors in the embryonic telencephalon are divided into regional domains with distinct developmental potentials by regional transcription factor expression (Flames et al., 2007). *Nkx2.1* encodes a transcription factor and is expressed in the ventral-most region of the telencephalon including the medial ganglionic eminence (MGE), preoptic area, and anterior entopeduncular region (Flames et al., 2007; Puelles et al., 2000). *Nkx2.1*-expressing precursors give rise to a diverse assortment of neurons in the globus pallidus, cerebral cortex, striatum, and hippocampus (Butt et al., 2008; Flandin et al., 2010; Xu et al., 2008).

Shh signaling is required to induce and maintain the expression of *Nkx2.1* in the embryonic brain. Using a *FoxG1*-Cre driver to delete a conditional *Smo* allele throughout the telencephalon at E9, Fishell and colleagues find that the expression of ventral genes including *Nkx2.1* is almost entirely absent from the ventral telencephalon (Fuccillo et al., 2004). Deletion of *Smo* (Machold et al., 2003), or *Shh (Xu et al., 2005)* at ~E10.5 resulted in the significant loss of *Nkx2.1* expression in the ventral telencephalon. Moreover, inhibition of Shh pathway activity by the SMO antagonist cyclopamine resulted in the significant loss of *Nkx2.1* expression in the

MGE (Gulacsi and Anderson, 2006). Thus, Shh signaling is important for both the establishment and maintenance of the *Nkx2.1* domain in the ventral embryonic telencephalon.

Section 3: Neural precursor diversity in the adult brain

Neurons continue to be generated in the adult brain within the ventricular-subventricular zone (V-SVZ) along the walls of the lateral ventricle (Lim and Alvarez-Buylla, 2014). Neural precursors in the V-SVZ generate a diverse assortment of olfactory bulb (OB) interneurons throughout life (Lledo et al., 2008). Precursors in different regions of the V-SVZ generate distinct OB interneuron subtypes (Ihrie et al., 2011; Merkle et al., 2007). Like the embryonic telencephalon, the adult V-SVZ is regionally patterned by transcription factor expression which is thought to underlie regional differences in V-SVZ developmental potential (Merkle et al., 2014). Interestingly, the adult V-SVZ is patterned with many of the same transcription factors found in the embryonic brain, including *Nkx2.1* which is found ventrally(Merkle et al., 2014). Furthermore, fate-labeling experiments reveal that RGCs in the early postnatal brain give rise to V-SVZ precursors (Merkle et al., 2004). Taken together, these data suggest that the positional identity of V-SVZ NSCs is established in embryogenesis. However it is now known whether *Nkx2.1*+ V-SVZ precursors arise from *Nkx2.1*+ precursors in the embryo.

While Shh signaling is present in the adult V-SVZ (Balordi and Fishell, 2007a, b; Traiffort et al., 1999), the role it plays in fate specification is unknown. Deletion of *Shh* in adulthood resulted in the decreased production of OB interneurons with ventral fate, suggesting that Shh signaling is still required to maintain the identity of ventral neural precursors in adulthood (Ihrie et al., 2011). However, transplantation studies performed by Merkle et al. (2007) found that ventral V-SVZ precursors retained their developmental potential when transplanted to the dorsal V-SVZ, away from high levels of Shh signaling. Furthermore, the effects of Shh signaling on transcription factor expression in the postnatal V-SVZ remain unknown. Is Shh signaling required to maintain *Nkx2.1* expression in the postnatal V-SVZ?

CHAPTER 2: Embryonic *Nkx2.1*-expressing neural precursor cells contribute to the regional heterogeneity of adult V-SVZ neural stem cells

SUMMARY

The adult ventricular-subventricular zone (V-SVZ) of the lateral ventricle produces several subtypes of interneurons for the olfactory bulb (OB) throughout life. Neural stem cells (NSCs) of the V-SVZ are regionally heterogeneous, with NSCs located in different regions of the lateral ventricle wall generating different OB interneuron subtypes. The regional expression of specific transcription factors appears to correspond to such geographical differences in the developmental potential of V-SVZ NSCs. However, the transcriptional definition and developmental origin of V-SVZ NSC regional identity are not well understood. In this study, I found that a population of NSCs in the ventral region of the V-SVZ expresses the transcription factor NKX2.1 and is derived from *Nkx2.1*-expressing (*Nkx2.1*+) embryonic precursors. To follow the fate of *Nkx2.1*+ cells *in vivo*, I used mice with an *Nkx2.1*-CreER "knock-in" allele. *Nkx2.1*+ V-SVZ NSCs labeled in adult mice generated interneurons for the deep granule cell layer of the OB. Embryonic brain *Nkx2.1*+ precursors labeled at embryonic day 12.5 gave rise to *Nkx2.1*+ NSCs of the ventral V-SVZ in postnatal and adult mice. Thus, embryonic *Nkx2.1*+ neural precursors give rise to a population of *Nkx2.1*+ NSCs in the ventral V-SVZ where they contribute to the regional heterogeneity of V-SVZ NSCs.

INTRODUCTION

The adult ventricular-subventricular zone (V-SVZ) harbors neural stem cells (NSCs) that are distributed throughout the walls of the lateral ventricles. While the V-SVZ as a whole produces numerous subtypes of olfactory bulb (OB) interneurons, V-SVZ NSCs are not equivalent (Lim and Alvarez-Buylla, 2014; Lledo et al., 2008). For instance, NSCs in the dorsal V-SVZ give rise to OB interneuron subtypes that are distinct from ventral V-SVZ NSCs (Ihrie et al., 2011; Merkle et al., 2007). Differences in V-SVZ NSC developmental potential appear to relate to regional transcription factor expression that divides the V-SVZ into numerous domains (Lim and Alvarez-Buylla, 2014; Lledo et al., 2008; Lopez-Juarez et al., 2013; Merkle et al., 2014). However, the gene expression that defines the different populations of V-SVZ NSCs is not well known, and it is also not clear how such regional differences are established.

The adult V-SVZ exhibits regional patterns of transcription factor expression similar to those observed in embryonic development, suggestive of a lineage relationship between region-specific embryonic neural precursors and NSCs of the adult V-SVZ (Alvarez-Buylla et al., 2008). For instance, the dorsal V-SVZ expresses *Gsh2* (Lopez-Juarez et al., 2013) and other transcription factors of the lateral ganglionic eminence (LGE) (Kohwi et al., 2005; Waclaw et al., 2006). While the *Gsh2*-Cre transgene drives recombination in many adult V-SVZ cells and OB interneurons (Young et al., 2007), constitutive Cre-drivers do not generally establish a precise time at which cell labeling occurred.

The most compelling evidence for an embryonic origin of V-SVZ regional identity relates to the most dorsal V-SVZ NSC population. Using *Emx1*-CreER mice to label a cohort of E10.5 neural precursors in the developing pallium, Young et al. (2007) showed this population gives rise to adult NSCs in the dorsal V-SVZ.

In contrast, the contribution of *Nkx2.1*-expressing (*Nkx2.1*+) neural precursors to OB neurogenesis has been unclear. Cells from the medial ganglionic eminence (MGE) – but not

those of the LGE or pallium – express *Nkx2.1*, and *in utero* fate-tracing analysis suggests that E13.5 MGE cells do not normally generate OB interneurons. However, E13.5 MGE cells transplanted into the adult V-SVZ produce a small number of neurons located in the OB (Wichterle et al., 1999). In embryonic *Nkx2.1*-Cre mice, a small stream of labeled cells is observed migrating from the MGE to the OB (Xu et al., 2008). However, very few *Nkx2.1*-Cre-labeled OB neurons are born in adult mice (Young et al., 2007). Using a tamoxifen-inducible *Nkx2.1*-CreER transgene to follow the fate of early postnatal *Nkx2.1*+ cells, Merkle and colleagues also observed very few labeled OB neurons (Merkle et al., 2014). These results, taken together, have suggested that *Nkx2.1*+ neural precursors do not contribute substantially to the adult NSC population and OB neurogenesis. Furthermore, these previous studies did not address the embryonic origin of the *Nkx2.1*+ V-SVZ NSC population.

In this report, I show that postnatal and adult *Nkx2.1*+ V-SVZ cells in the ventral brain generate OB interneurons throughout life and derive from embryonic *Nkx2.1*+ precursors. These data indicate that embryonic *Nkx2.1*+ cells give rise to a regionally distinct population of V-SVZ NSCs in the adult brain, providing clear evidence that the ventral identity of neural precursors established in the early embryonic brain persists throughout development and into adulthood.

MATERIALS AND METHODS

Animal Husbandry and Procedures

All experiments were performed with mice of either sex and in accordance to protocols approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco. C57BL/6 (wild type) mice were obtained from Jackson Laboratories. Mice homozygous for Ai14 (Madisen et al., 2010) were mated to mice with the *Nkx2-1*tm1.1(cre/ERT2)Zjh allele (Taniguchi et al., 2011). For the purpose of adult fate-tracing, >P60 mice received 5 mg tamoxifen (Sigma) dissolved in corn oil (Sigma) by oral gavage per 30 grams of body weight once a day for 5 consecutive days. For embryonic fate-tracing, timed pregnant dams with *Nkx2.1*-CreER; Ai14 embryos were given one dose of tamoxifen (5mg per 30g body weight) by oral gavage. Embryonic day 0.5 (E0.5) was estimated to be 12:00 pm on the day that vaginal plugs were observed. Bromodeoxyuridine (BrdU) (Sigma) was administered via drinking water (1mg/mL) for one week.

Immunohistochemistry

Embryonic brains were fixed in 4% PFA. Transcardiac perfusion was performed on postnatal and adult animals first with phosphate buffered saline (PBS) (pH 7.4) and subsequently with 4% PFA. For cryoprotection, I equilibrated brains in PBS with 30% sucrose. After equilibration, brains were then embedded in OCT (Tissue-Tek). 12 μm cryosections were cut at -20°C in a Microm HM 525 cryostat (Thermo Scientific) and stored at -80°C. Whole mount preparations were prepared as previously described (Mirzadeh et al., 2010). Immunohistochemistry (IHC) was performed with protocols as previously described (Hwang et al., 2014). Antigen-retrieval for BrdU IHC was performed using 2N HCL (Tang et al., 2007). Primary antibodies used in this study include: BrdU (rat, 1:250, Abcam), Dcx (rabbit, 1:500, Abcam), Dcx (guinea pig, 1:500, Millipore), Dlx2 (guinea pig, 1:2000, a kind gift from Dr. Kazuaki Yoshikawa), dsRed (rabbit, 1:1000, Living Colors), GFAP (chicken, 1:1000, Abcam), Ki67 (rabbit, 1:500, Vector), Nestin

(chicken, 1:500, Aves), Nkx2.1 (rabbit, 1:400, Santa Cruz), Nkx2.1 (mouse IgG1, 1:250, Leica), Phospho-vimentin, Ser55 (mouse IgG2b, 1:500, MBL), RFP (rat, 1:1000, Chromotek), Sox2 (goat, 1:200, Santa Cruz), S100β (mouse, 1:500, Sigma). Alexfluor conjugated secondary antibodies (Life Technologies) were used at 1:500. Nuclear counterstain DAPI (Sigma) was applied at 1:100000.

Imaging and quantification

Imaging was performed using either a DMI4000 B epifluorescent microscope with attached DFC345 FX camera or a TCS SP5 X confocal microscope (Leica). Image processing including cropping and pseudo-coloring was performed with Fiji (Schindelin et al., 2012) and Photoshop CC (Adobe). Quantification of tdTomato+ cells per unit area was performed using Fiji. Briefly, the border of the granular cell layer (GCL) was traced in Fiji using the “Freehand Selection” tool and the area was then calculated using the “Measure” tool. Student’s t-tests were performed using Prism 6 (Graph Pad).

RESULTS

NKX2.1 is expressed in the ventral germinal zone of the embryonic and early postnatal telencephalon

To determine the temporospatial expression of NKX2.1 in embryonic and postnatal telencephalic germinal zones, I performed immunohistochemical (IHC) analysis of coronal brain sections from E12.5, E15.5, P0, and P7 mice. *Nkx2.1* is expressed in a restricted region of the early neural tube starting at around E9 (Price et al., 1992; Shimamura et al., 1995; Sussel et al., 1999). Consistent with previous results (Flames et al., 2007), I observed *Nkx2.1* expression throughout the E12.5 MGE (Figure 2.1*A*), with this domain of expression ending abruptly in the sulcus between the MGE and LGE (Figure 2.1*A*'). Shortly after E14.5 the ganglionic eminences flatten, forming the lateral ventricle wall. At E15.5, NKX2.1+ cells were present in a ventral domain along both the medial and lateral walls of the lateral ventricle (Figures 2.1*B*,*B'*). This ventral domain was also present postnatally at P0 and P7 (Figures 2.1*C*,*D*). At all ages analyzed, the NKX2.1+ domain had distinct dorsal borders in both the medial and lateral walls (Figures 2.1*A'*-*D'*).

Radial glial cells (RGCs) are the primary neural precursor of the embryonic brain (Kriegstein and Alvarez-Buylla, 2009). At E12.5 and E15.5, many of the NKX2.1+ cells close to the ventricle wall exhibited typical RGC characteristics, including a long radial process and the expression of Nestin (Figures 2.2*A*,*B*). Some NKX2.1+ cells expressed phospho-vimentin (Figures 2.2*I*,*J*), which is detected in mitotic RGCs. At the early postnatal ages (P0 and P7), many NKX2.1+ cells were also Nestin+ (Figures 2.2*C*,*D*) and proliferative, as evidenced by the expression of Ki67 (Figures 2.2*G*,*H*) and Phospho-Vimentin (Figures 2.2*K*,*L*). Thus, proliferative NKX2.1+ neural precursor cells exist in the ventral telencephalon through early postnatal development.

Adult *Nkx2.1*+ V-SVZ cells are neurogenic *in vivo*

IHC of adult (>P60) brains revealed a domain of NKX2.1+ cells at the ventral V-SVZ (Figure 2.3*A*). NKX2.1 immunoreactivity was not observed in the dorsal V-SVZ. The majority of NKX2.1+ cells corresponded to ependymal cells expressing S100β (Figure 2.3*B*). Type B1 cells – the V-SVZ NSC population – do not express S100β but are immunopositive for glial fibrillary acidic protein (GFAP) (Didier et al., 1986; Doetsch et al., 1997; Mirzadeh et al., 2008). A small proportion of NKX2.1+ cells were negative for S100β but were immunopositive for GFAP (Figure 2.3*B*). Type B1 cells characteristically also have endfeet upon blood vessels (Mirzadeh et al., 2008), and I observed GFAP+/NKX2.1+ cells with processes contacting blood vessels (Figure 2.3*C*). Some NKX2.1+ cells were also Ki67+ (Figure 2.3*D*), suggesting that these NKX2.1+ cells continue proliferating in adulthood. I also observed NKX2.1+ cells that co-expressed markers of the V-SVZ neurogenic lineage (Figure 2.3*E*) including DLX2, a key transcription factor for the genesis and migration of interneurons (Long et al., 2007), and DCX, a marker of migratory neuroblasts. After being born in the V-SVZ, DCX+ neuroblasts migrate anteriorly along the lateral ventricle in chains that merge into the rostral migratory stream (RMS) before entering the core of the OB (Doetsch and Alvarez-Buylla, 1996; Doetsch et al., 1999; Lois and Alvarez-Buylla, 1994; Luskin, 1993). I observed DCX+/NKX2.1+ cells within the RMS in the rostral telencephalon (Figure 2.3*F*), suggesting that NKX2.1+ neuroblasts born in the V-SVZ follow the same migratory path as other adult-born OB interneurons. Together, these data indicated that *Nkx2.1* is expressed in V-SVZ cells of the adult neurogenic lineage.

MGE-born cortical interneurons down-regulate *Nkx2.1* expression prior to arriving in the cortex (Marin et al., 2000; Nobrega-Pereira et al., 2008). Similarly, I did not detect any NKX2.1 immunopositive cells within the OB (Figure 2.3*G*). To investigate whether *Nkx2.1*+ cells in the adult brain generate OB interneurons, I employed a genetic fate-tracing strategy with the *Nkx2.1*-CreER allele, in which the CreER expression cassette is inserted into the endogenous *Nkx2.1* locus (Taniguchi et al., 2011). Administration of tamoxifen to *Nkx2.1*-CreER mice

induces Cre-mediated recombination in *Nkx2.1*-expressing cells and their descendants (Taniguchi et al., 2011). To follow the fate of cells that undergo Cre-mediated recombination, I used the Ai14 transgene, which expresses tdTomato after excision of a "floxed-stop" cassette (Madisen et al., 2010). I administered tamoxifen to *Nkx2.1*-CreER; *Ai14* animals from P60-64 and analyzed brains 4 weeks (wk) later (Figure 2.4*A*). After 5 d of tamoxifen administration, I observed tdTomato+ cells in the ventral V-SVZ (data not shown), but not the OB (Figure 2.4*B*). After 4 wk, I observed tdTomato+ cells along the ventral tip of the lateral ventricles (Figures 2.4*C*,*D*) consistent with the regional expression of NKX2.1 protein (Figure 2.3*A*). To determine the identity of the tdTomato+ (hereafter referred to as *Nkx2.1*^{P60}-labeled) cells, I performed IHC for the type B1 cell marker GFAP, and for the ependymal cell marker S100β. I found that many *Nkx2.1*^{P60}-labeled cells expressed S100β and had the morphology of ependymal cells. However, some *Nkx2.1*^{P60}-labeled cells were located immediately below the ependymal cell layer and expressed GFAP (Figure 2.4*C*), suggesting a type B1 cell identity. I also observed DCX+ / *Nkx2.1*^{P60}-labeled cells within migratory chains along the walls of the V-SVZ (Figure 2.4*D*) indicating that *Nkx2.1*+ cells give rise to cells of the V-SVZ neurogenic lineage.

After arriving into the OB, young neuroblasts exit the RMS and begin differentiating into interneurons. This progressive maturation of newly-born neurons can be categorized into five stages or (1-5) based on their location and morphology (Petreanu and Alvarez-Buylla, 2002). Stage 1 cells are the most immature, having the morphology typical of neuroblasts 5-7 d after their migration from the V-SVZ and located primarily in the OB core, which is the most rostral extent of the RMS (Petreanu and Alvarez-Buylla, 2002). 17% (18/103) of *Nkx2.1*^{P60}-labeled cells were class 1 (Figure 2.4*E*), suggesting that these cells had recently arrived from their origin in the adult V-SVZ. Class 4/5 cells are more mature, having elaborate branched apical dendrites, and represent 11-30 days of differentiation (Petreanu and Alvarez-Buylla, 2002). I observed class 4/5 tdTomato+ cells in 3 of 3 animals (Figure 2.4*F*), suggesting that *Nkx2.1*^{P60}-

labeled V-SVZ cells give rise to mature OB interneurons. The majority of tdTomato+ cells were located deep in the GCL (0.87 tdTomato+ cells/mm^2 s.d. 0.43, n=3, p<0.005, Figure 2.4*G*,*H*). I did not observe tdTomato+ cells that co-expressed GCL markers CalR or NPY (data not shown). tdTomato+ periglomerular cells (PGC) were also observed, but were exceptionally rare (2 cells in 90 12um sections). Taken together, these data indicate that *Nkx2.1*+ adult V-SVZ precursors generate substantial numbers of new deep neurons for the OB GCL in adulthood.

Postnatal and adult NKX2.1+ NSCs are derived from early embryonic *Nkx2.1*+ neural precursors

I next investigated whether embryonic *Nkx2.1*+ neural precursors give rise to the ventral NKX2.1+ NSCs of the postnatal V-SVZ. To follow the fate of embryonic *Nkx2.1*-expressing precursors into early postnatal brain development, I administered tamoxifen at E12.5 to *Nkx2.1*-CreER; Ai14 mice and analyzed brains (n=3) at P7 (Figure 2.5*A*). Consistent with previous results (Taniguchi et al., 2011), I observed tdTomato+ (hereafter referred to as *Nkx2.1*$^{E12.5}$-labeled) cells in the ventral germinal zone of the late embryonic brain. At P7, *Nkx2.1*$^{E12.5}$-labeled cells were present in the ventral V-SVZ (Figure 2.5*B*). *Nkx2.1*$^{E12.5}$-labeled cells in the V-SVZ were restricted to the ventral tip in a pattern similar to that of NKX2.1 protein expression (Figures 2.1*D*,*D'*). To determine whether *Nkx2.1*-labeled cells from E12.5 still express NKX2.1 protein at P7, I performed co-IHC for tdTomato and NKX2.1 (Figure 2.5*C*). 99.6%, (286 of 287 cells) of the *Nkx2.1*$^{E12.5}$-labeled cells were also NKX2.1+.

At around P7, type B1 cells emerge and begin to express GFAP (Merkle et al., 2004). In the P7 brain, I observed GFAP+ / *Nkx2.1*$^{E12.5}$-labeled cells in the ventral V-SVZ (Figure 2.5*D*). DCX+ / *Nkx2.1*$^{E12.5}$-labeled cells were also observed among chains in the ventral, middle and dorsal V-SVZ regions (Figure 2.5*E*). These data suggest that *Nkx2.1*$^{E12.5}$-labeled precursors remain neurogenic in the postnatal brain.

Next I sought to determine whether *Nkx2.1*-expressing embryonic precursors also

contribute NSCs to the adult V-SVZ. For this, I analyzed P60 mouse brains (n=3) for *Nkx2.1*$^{E12.5}$-labeled cells. As in the P7 animals, *Nkx2.1*$^{E12.5}$-labeled cells in the V-SVZ of P60 brains were restricted to the ventral tip of the lateral ventricle (Figure 2.5*F*). IHC revealed that 99.5% (219 of 220) of *Nkx2.1*$^{E12.5}$-labeled cells in the V-SVZ were also NKX2.1+ (Figure 2.5*G*). Ependymal cells arise from ventricle-contacting precursors during early postnatal development (Spassky et al., 2005) and constitute a significant proportion of the V-SVZ (Doetsch et al., 1997; Mirzadeh et al., 2008). As expected, the majority of *Nkx2.1*$^{E12.5}$-labeled cells corresponded to S100β+ ependymal cells (Figure 2.5*H*). However, a small number of *Nkx2.1*$^{E12.5}$-labeled cells expressed GFAP (Figure 2.5*I*), suggestive of a type B1 cell identity. As in the P7 brains, I also observed DCX+ / *Nkx2.1*$^{E12.5}$-labeled cells among chains of neuroblasts in the lateral ventricle walls (Figure 2.5*J*) as well as in the RMS (Figures 2.5*K*,*L*). To confirm that *Nkx2.1*$^{E12.5}$-labeled cells in the RMS were born in the adulthood, I continuously administered BrdU to the mice for 1 wk prior to analysis. IHC revealed that *Nkx2.1*$^{E12.5}$-labeled cells in the RMS incorporated BrdU (Figure 2.5*K*), strongly suggesting that they had been born in adulthood. In the OB of P60 brains, there were numerous *Nkx2.1*$^{E12.5}$-labeled cells in the GCL (Figure 2.5*M*), but very few in the periglomerular layer. While the majority (94%, 1346 of 1447) of the *Nkx2.1*$^{E12.5}$-labeled cells in the GCL of P60 brains already had mature neuronal morphologies, 6% (101 of 1447) of the *Nkx2.1*$^{E12.5}$-labeled cells were present in the OB core (Figure 2.5*M*) and had Stage 1 neuroblast morphologies, suggesting that some of these cells had been recently born in adulthood. Taken together, these data support a model in which early embryonic MGE neural precursors give rise to a population of adult V-SVZ NSCs that take residence in a restricted ventral domain and generate new OB neurons throughout adult life (Figure 2.6).

DISCUSSION

My results indicate that the ventral V-SVZ contains a population of *Nkx2.1*+ NSCs that continue to generate OB neurons throughout life. These NKX2.1+ cells were derived from *Nkx2.1*-expressing cells in the early embryonic brain, indicating that ventral germinal zones give rise to a regional population of adult NSCs, contributing to the regional heterogeneity of the adult V-SVZ.

Nkx2.1+ V-SVZ NSCs primarily generated OB interneurons of the deep granule cell layer (GCL). OB granule cells can be classified into three subtypes depending on the position of their cell body within the GCL: superficial, intermediate, or deep (Price and Powell, 1970). While NSCs in the dorsal V-SVZ give rise to superficial granule cells, NSCs in the ventral V-SVZ primarily generate deep granule cells (Merkle et al., 2007). The ventral location of NKX2.1+ NSCs within the V-SVZ (Figure 2.3) is therefore consistent with their production of deep OB granule cells. V-SVZ NSCs also have a rostral-caudal identity. While rostral V-SVZ NSCs produce many PGCs, the caudal V-SVZ produces very few (Merkle et al., 2007). Consistent with the caudal location of the *Nkx2.1* domain within the V-SVZ (Figure 2.3 and (Merkle et al., 2014), I observed very few PGCs born from adult *Nkx2.1*+ NSCs. Therefore, the *Nkx2.1* V-SVZ domain generated neurons consistent with a ventral, caudal NSC identity.

In comparison to the dorsal-lateral regions of the V-SVZ, there are relatively few NSCs in the ventral regions of the lateral ventricle (Mirzadeh et al., 2008). Moreover, the adult *Nkx2.1*+ domain (Figure 2.3) is a small proportion of the entire V-SVZ. Thus, the number of *Nkx2.1*-lineage OB interneurons born in the adult mouse brain (Figure 2.4) appears to be commensurate with the relatively small size of the *Nkx2.1* V-SVZ domain and the paucity of NSCs in this ventral region. Given that *Nkx2.1*+ NSCs continued to generate new OB neurons late into life, it is interesting to consider that the neurons generated by this spatially restricted population of NSCs might have unique and/or important functions for olfaction.

Previous studies observed a much more limited extent of neurogenesis from *Nkx2.1*+

cells in the postnatal (Merkle et al., 2014) and adult V-SVZ (Young et al., 2007). Differences in Cre-driver expression and efficiency are the simplest and most likely explanation for this disparity in results. Transgene expression can differ significantly from endogenous gene expression (Giraldo and Montoliu, 2001). The P1 bacteriophage-derived artificial chromosomes (PAC)-based Cre drivers used by Merkle et al. (2014) and Young et al. (2007) (described in (Kessaris et al., 2006; Tsai et al., 2012)) may not include all of the key genomic regulatory elements involved in *Nkx2.1* expression, and the site of integration can also affect the efficiency of transgene expression. Thus, the PAC-based Cre drivers may not label *Nkx2.1*-expressing cells as effectively as the "knock in" *Nkx2.1*-CreER allele used in my study.

What is the developmental origin of the different V-SVZ NSC populations? While *Emx1+* embryonic neural precursors of the developing cortex have been fate-traced to adult NSCs in the dorsal V-SVZ, the origin of NSCs in other regions of the adult lateral ventricle has been less clear. Multiple studies have implicated the LGE as an important origin of V-SVZ NSCs (Kohwi et al., 2005; Waclaw et al., 2006; Young et al., 2007). For example, E13.5 LGE cells produce many OB neurons during development (Wichterle et al., 2001), and when grafted into the adult brain, LGE cells can also generate neurons for the OB (Wichterle et al., 1999). Furthermore, the embryonic LGE and MGE express *Gsh2*, and adult *Gsh2*-Cre mice have labeled NSCs in the V-SVZ (Young et al., 2007). However, because *Gsh2* is also expressed in the adult V-SVZ (Lopez-Juarez et al., 2013), the *Gsh2*-Cre transgene may label V-SVZ cells in both the embryonic and adult brain, making it difficult to establish an embryonic origin to the *Gsh2*+ cells of the adult V-SVZ. Similarly, while *Nkx2.1*-Cre labels cells in the ventral V-SVZ (Young et al., 2007), I found *Nkx2.1* to be expressed throughout development and into adulthood (Figures 2.5*C*,*G*), again making it unclear when *Nkx2.1*-Cre induces recombination.

To more clearly establish a temporal relationship between a population of embryonic neural precursors and V-SVZ NSCs, I used the *Nkx2.1*-CreER allele to label a cohort of *Nkx2.1*+ precursors at E12.5, which gave rise to NSCs of the postnatal and adult V-SVZ. At

E12.5, the expression of *Nkx2.1* distinguishes the MGE from the LGE (Flames et al., 2007). While *Nkx2.1* is expressed in other E12.5 ventral brain regions such as the septum and preoptic area (POA), the MGE does not express *Dbx1,* which is detected in these other ventral embryonic regions (Hirata et al., 2009). Mice with the *Dbx1*-Cre transgene do not appear to have labeled cells in the ventral V-SVZ (Young et al., 2007). Thus, in light of these other studies, my fate-tracing analysis of embryonic *Nkx2.1*+ cells suggests that the MGE is an important embryonic origin to the NSCs of the ventral V-SVZ.

Do transcription factors that determine regional identity in the embryonic brain continue to define developmental differences in V-SVZ NSCs of the adult? *Gsh2* is expressed in neural precursors in the LGE and MGE, and the *Gsh2*-Cre driver labels cells that later become V-SVZ NSCs that are distributed along the entire dorsal-ventral extent of the lateral ventricle wall (Young et al., 2007). However, using both a *Gsh2*-GFP reporter mouse and GSH2 IHC analysis to detect active *Gsh2* transcription, Nakafuku and colleagues found that *Gsh2* expression in the adult V-SVZ is restricted to the dorsolateral regions and is not detected in cells in the ventral V-SVZ (Lopez-Juarez et al., 2013). These results indicate that while a large proportion of the V-SVZ derive from cells that express *Gsh2* at some point during development, *Gsh2* expression is not maintained in most cells of the *Gsh2*-lineage. In contrast, embryonic *Nkx2.1*+ neural precursors give rise to a much more spatially restricted population of V-SVZ NSCs (Young et al., 2007), and virtually every *Nkx2.1*$^{E12.5}$-labeled cell in the V-SVZ continued to express NKX2.1 protein in the postnatal and adult brain (Figure 2.5). RGCs are considered to be the NSC population of the embryonic brain (Kriegstein and Alvarez-Buylla, 2009). In addition to generating many neurons and glia during embryogenesis, RGCs also give rise to V-SVZ NSCs during early postnatal development (Merkle et al., 2004). Taken together, my results suggest that the expression of *Nkx2.1* in embryonic RGCs is “maintained” as they continue dividing throughout development and after become NSCs of the adult brain (Figure 2.6).

The regional identity of neural precursors is a critical aspect of normal brain development. How is *Nkx2.1* expression maintained in a spatially precise pattern despite the rapid growth and the complex anatomical changes of the developing brain? While sonic hedgehog (SHH) signaling is required for the expression of *Nkx2.1* and many other ventral transcription factors through mid-embryonic development (Fuccillo et al., 2004; Gulacsi and Anderson, 2006; Hebert and Fishell, 2008; Xu et al., 2005), it is unclear whether neural precursors require SHH-signaling to maintain the expression of these transcription factors later in development. Future studies may determine the extent to which external morphogens such as SHH and cell-intrinsic epigenetic mechanisms maintain the expression of important determinants of neural precursor regional identity.

CONCLUSIONS

In conclusion, I provide clear evidence that *Nkx2.1*-expressing precursors in the E12.5 embryo give rise to a specific regional population of V-SVZ NSCs that maintain *Nkx2.1* expression throughout life. Consistent with their ventral position within the V-SVZ, *Nkx2.1*+ V-SVZ precursors generate a subpopulation of deep granule interneurons. Further studies regarding how *Nkx2.1* expression is regulated over time will provide key insights into how NSC heterogeneity is maintained throughout development.

Figure 2.1

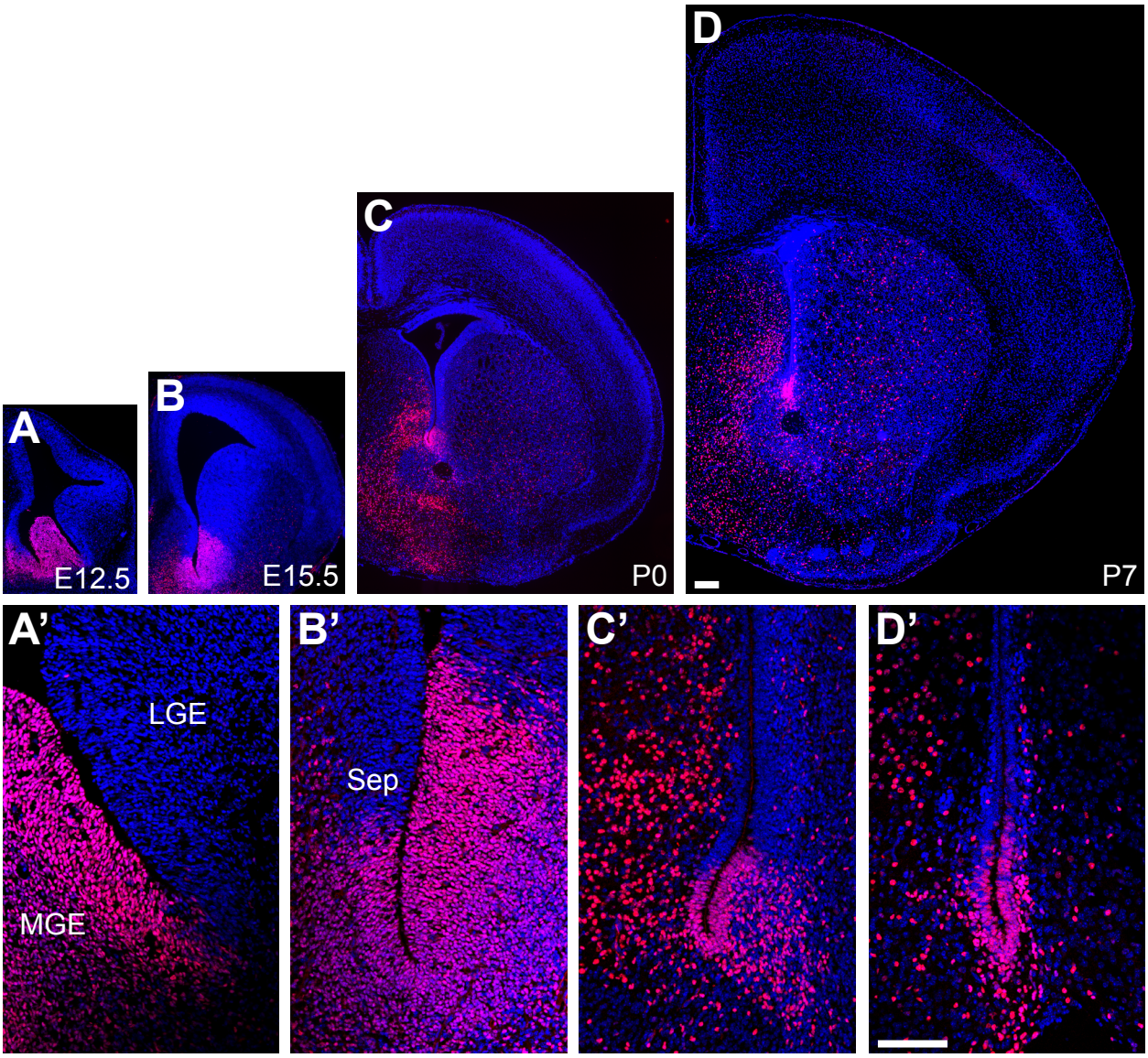


Figure 2.1. NKX2.1+ is expressed in ventral germinal regions throughout development. ***A-D***, IHC analysis of coronal brain sections for NKX2.1 (red) and DAPI (blue) from mice at low power magnification at E12.5 (***A, A'***), E15.5 ***(B, B')*** P0 **(*C, C'*)** P7 (***D, D'***). ***A'-D'***, higher-magnification confocal images of ***A-D***. Scale bars=100 μm.

Figure 2.2

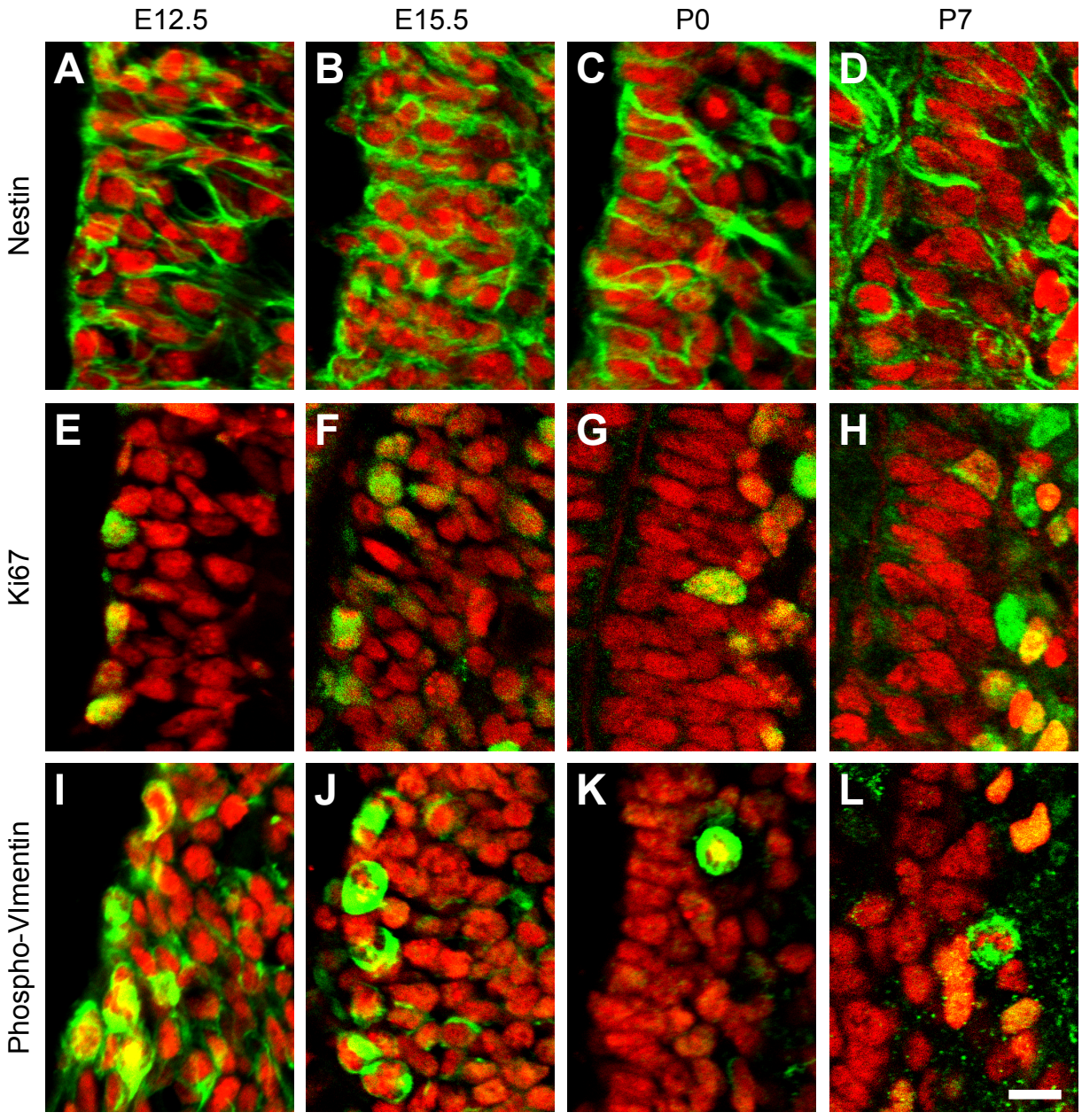


Figure 2.2. NKX2.1+ cells in the embryonic and postnatal brain have neural precursor cell identity. ***A-L***, IHC analysis of the ventral telencephalon for NKX2.1 (red) and neural precursor markers Nestin (***A-D***), Ki67 (***E-H***), and Phospho-Vimentin (***I-L***) (all in green) at E12.5 (***A,E,I***), E15.5 (***B,F,J***), P0 (***C,G,K***), and P7 (***D,H,L***). Scale bar=10 μm.

Figure 2.3

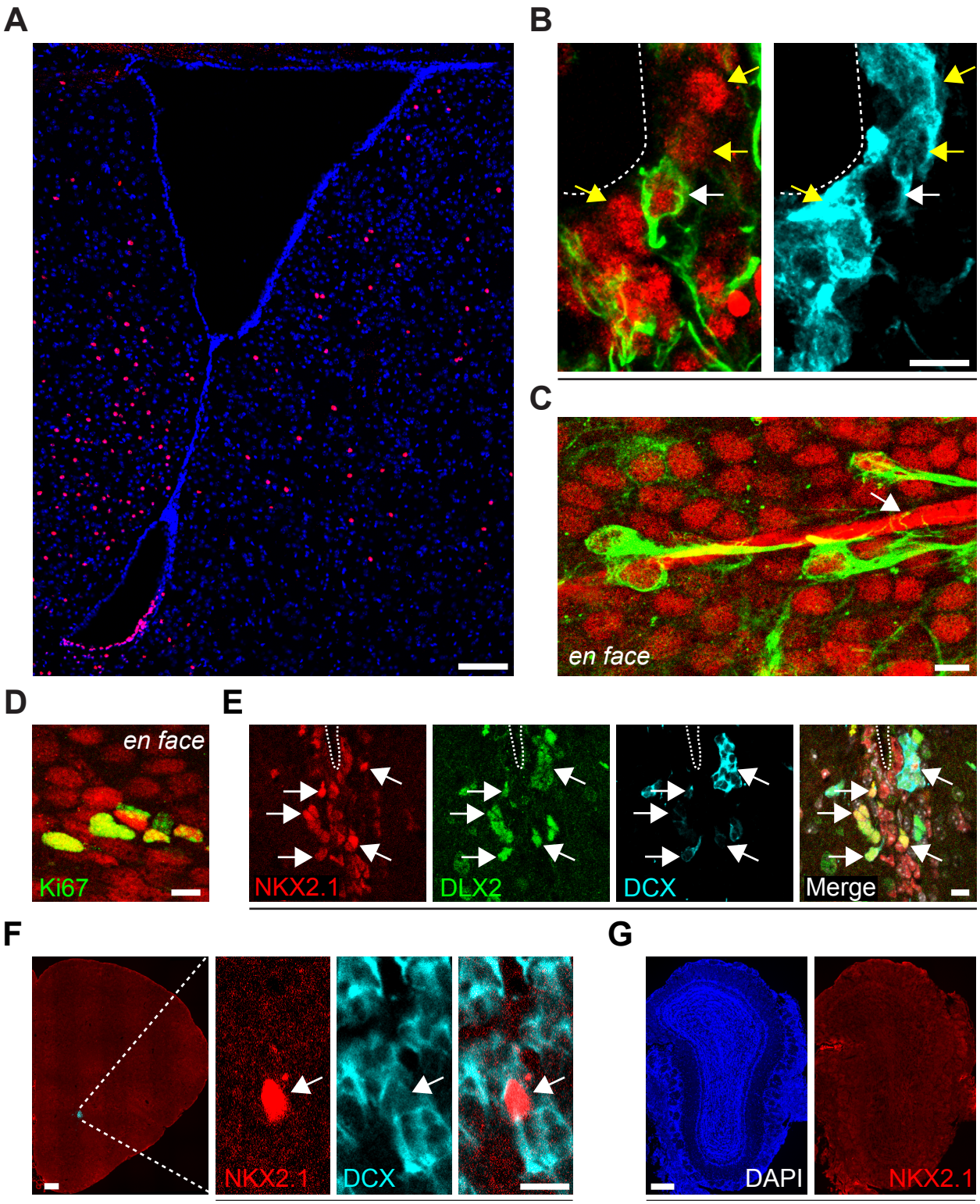
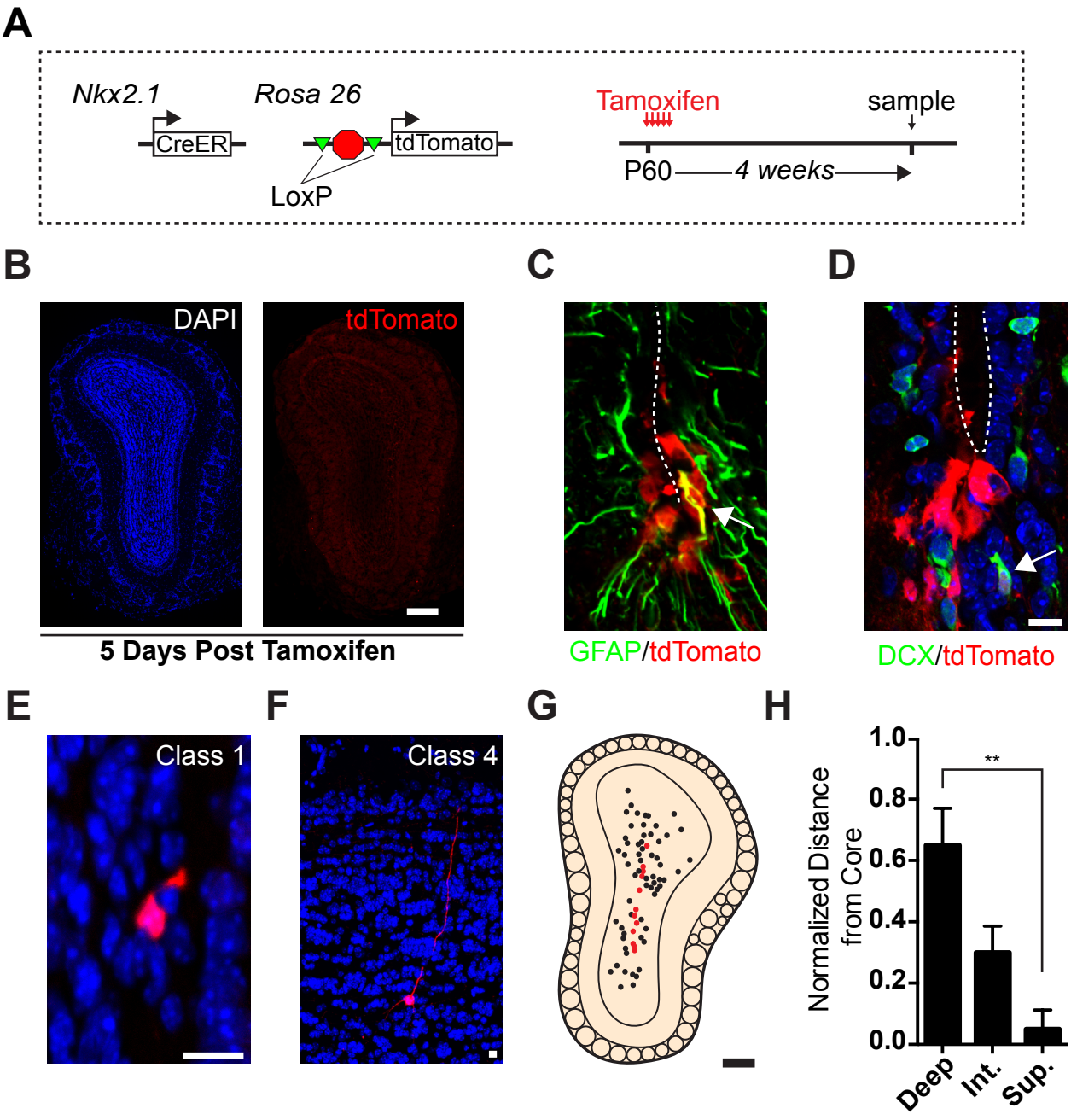

A
B
C
en face
D
en face
Ki67
E
NKX2.1
DLX2
DCX
Merge
F
NKX2.1
DCX
G
DAPI
NKX2.1

Figure 2.3. NKX2.1 is expressed in cells of the adult V-SVZ neurogenic lineage. ***A*****,** IHC analysis of P60 coronal brain sections for NKX2.1 (red) and DAPI (blue). ***B*****,** High-magnification confocal analysis of NKX2.1 (red), GFAP (green) and S100β (cyan) expression in the P60 ventral V-SVZ. Both panels are from the same focal plane. White dashed lined depicts lateral ventricle. White arrow points to NKX2.1+/GFAP+/S100β- cell. Yellow Arrows point to NKX2.1+/GFAP-/S100β+ cells. ***C*****,** IHC analysis of whole mount preparations derived from P60 brain. *en face* view of NKX2.1 (red) and GFAP (green) expression in the ventral surface of lateral ventricle. White arrow points to basal process contacting a blood vessel (visible because goat-anti-mouse secondary antibody was used). ***D*****,** NKX2.1 (red) and Ki67 (green) expression in the ventral surface of the P60 lateral ventricle, shown *en face*. ***E*****,** IHC analysis of coronal P60 sections showing NKX2.1 (red), DLX2 (green), and DCX (cyan) in the ventral V-SVZ. White dashed lined depicts lateral ventricle. **F,** NKX2.1 (red) and DCX (cyan) expression in the RMS at P60. (**G**) IHC analysis of NKX2.1 (red) and DAPI (blue) in the P60 olfactory bulb. Scale bars: (***A,F,G***) 250 μm (***B-E, F inset***) 10 μm.

Figure 2.4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Rosa 26
tdTomato
LoxP
Tamoxifen
sample
P60
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B
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tdTomato
5 Days Post Tamoxifen
C
GFAP/tdTomato
D
DCX/tdTomato
E
Class 1
F
Class 4
G
H
Normalized Distance from Core
1.0
0.8
0.6
0.4
0.2
0.0
**
Deep
Int.
Sup.

Figure 2.4. *Nkx2.1*+ cells generate OB interneurons in the adult mouse brain. ***A*****,** Adult *in vivo* *Nkx2.1*-labeling experimental design. **B**, tdTomato expression in the OB after 5 days of Tamoxifen administration. ***C*****-F,** IHC analysis of coronal sections from *Nkx2.1*-CreER:Ai14 animals 4 wk after labeling. High-magnification images of $Nkx2.1^{P60}$-labeled (red) cells with (***C***) GFAP (green) and (***D***) DCX (green) expression in ventral V-SVZ. White dashed line depicts lateral ventricle. Representative images of (***E***) Class 1 and (***F***) class 4 $Nkx2.1^{P60}$-labeled (red) cells in the OB. ***G***, Composite drawing of $Nkx2.1^{P60}$-labeled (red) cells from 112 12μM coronal sections in the OB 4 wk after labeling. Red dots depict $Nkx2.1^{P60}$-labeled cells within the OB core. Black dots depict $Nkx2.1^{P60}$-labeled cells outside of the OB core. ***H*****,** Normalized distribution of $Nkx2.1^{P60}$-labeled cells in the GCL. Deep= 0.00-0.33, Intermediate= 0.34-0.67, and Superficial= 0.68-1.00 bins calculated based on relative postion of cell between OB core and mitral cell layer. **p<0.005 Scale bars: (***C-F***) 10 μm (**B**,***G***) 250 μm.

Figure 2.5

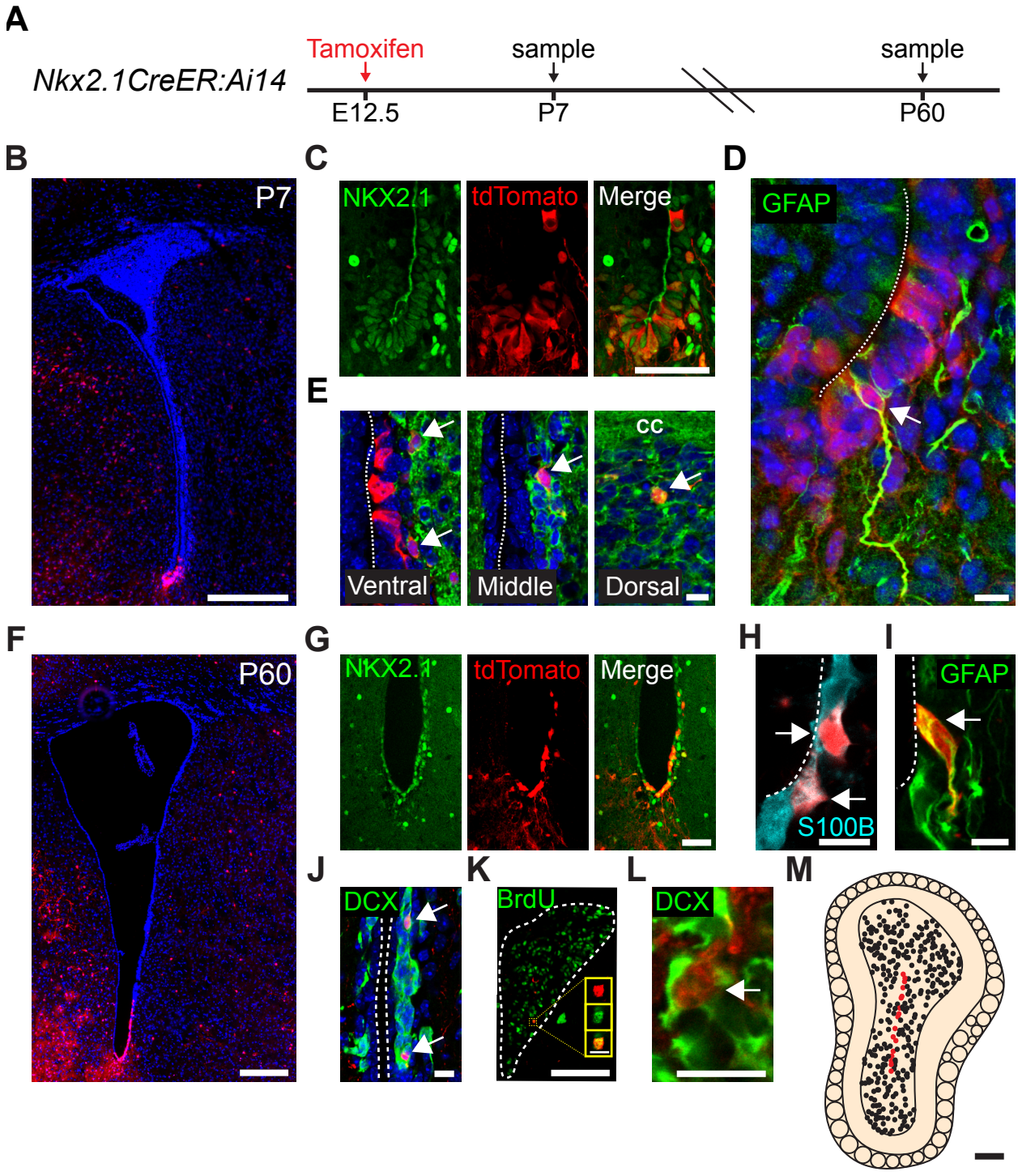
A
Nkx2.1CreER:Ai14
Tamoxifen
sample
sample
E12.5
P7
P60
B
P7
C
NKX2.1
tdTomato
Merge
D
GFAP
E
Ventral
Middle
Dorsal
cc
F
P60
G
NKX2.1
tdTomato
Merge
H
S100B
I
GFAP
J
DCX
K
BrdU
L
DCX
M

Figure 2.5. V-SVZ NKX2.1+ precursors arise from embryonic *Nkx2.1*+ precursors. ***A*****,** *Embryonic in vivo Nkx2.1*-labeling experimental design. ***B-E,*** *IHC analysis of coronal sections from* $Nkx2.1^{E12.5}$-labeled brains at P7. ***B***, Low-magnification image of $Nkx2.1^{E12.5}$-labeled (red) cells with DAPI (blue) counterstain at P7. High-magnification confocal images of $Nkx2.1^{E12.5}$-labeled (red) cells with (***C***) NKX2.1 (green) and (***D***) GFAP (green) expression in the ventral V-SVZ. ***E***, High-magnification confocal images of $Nkx2.1^{E12.5}$-labeled (red) cells and DCX (green) expression in the ventral, middle, and dorsal V-SVZ. ***F-M,*** *IHC analysis of coronal sections from* $Nkx2.1^{E12.5}$-labeled brains at P60. ***F***, Low-magnification image of $Nkx2.1^{E12.5}$-labeled (red) cells with DAPI (blue) counterstain at P60. High-magnification confocal images of $Nkx2.1^{E12.5}$-labeled (red) cells and (***G***) NKX2.1 (green), (***H***) S100β (cyan), and (***I***) *GFAP* (green) expression in the ventral V-SVZ at P60. White dashed lines depict lateral ventricle. ***J***, High-magnification image of $Nkx2.1^{E12.5}$-labeled (red) cells and DCX (green) expression in the lateral V-SVZ. White dashed lines depict lateral ventricle $Nkx2.1^{E12.5}$-labeled (red) cells in the RMS with (***K***) BrdU (green) and (***L***) DCX (green) IHC. White dashed line depicts border of RMS. ***M***, Composite drawing of $Nkx2.1^{E12.5}$-labeled cells from 25 12μM coronal sections in the P60 OB. Red dots depict $Nkx2.1^{E12.5}$-labeled cells within the OB core. Black dots depict $Nkx2.1^{E12.5}$-labeled cells outside of the OB core. Scale Bars: (***B,F,M***) 250 μm (***C-E***) 10 μm (***G***) 50 μm (***H-J,L***) 10 μm (***K***) 50 μm, inset 5 μm.

Figure 2.6

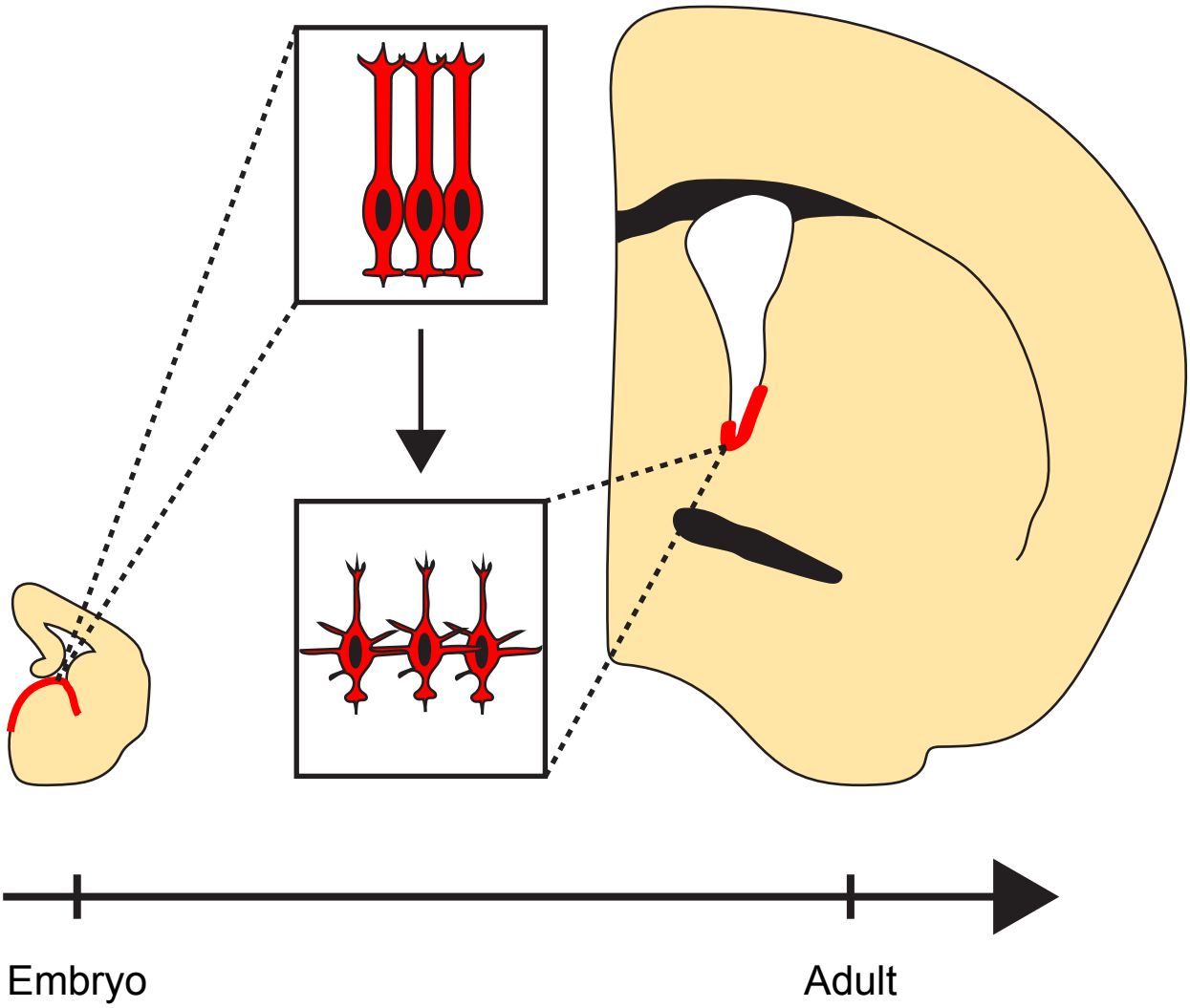

Figure 2.6. Regional identity of embryonic *Nkx2.1*+ precursors is maintained into adulthood. Model of *Nkx2.1*+ domain from embryogenesis into adulthood. *Nkx2.1*+ radial glia (depicted in upper inset) give rise to *Nkx2.1*+ type B1 cells (depicted in lower inset) of the adult V-SVZ.

Chapter 3: *Mixed-lineage leukemia 1* (*Mll1*) chromatin regulator maintains *Nkx2.1* expression in the postnatal mouse brain.

SUMMARY

Neural stem cells (NSCs) in both the embryonic and adult brain are divided into distinct domains by the regional expression of key developmental transcription factors, and such NSC positional identity is required for proper brain development. *Nkx2.1*-expressing NSCs in the embryonic brain persist throughout development and generate NKX2.1+ NSCs in the adult V-SVZ. It is unclear how *Nkx2.1* expression is maintained in the NSC population throughout this developmental timeline during which the brain grows substantially in size and anatomical complexity. During early embryonic brain development, Shh signaling is required for the establishment and maintenance of the ventral *Nkx2.1* NSC domain. However, at later stages of development, the role that extracellular signals play in NSC positional identity remains unclear. To investigate how *Nkx2.1* expression is regulated in the postnatal V-SVZ, I utilized an *in vitro* monolayer culture system that allowed for the long-term expansion of regional V-SVZ NSC populations. In this study, I found that postnatal V-SVZ NSCs do not require Shh signaling to maintain *Nkx2.1* expression. Instead, V-SVZ NSCs require *mixed-lineage leukemia 1 (Mll1),* a *trithorax* group (*trxG*) chromatin regulator, to maintain the expression of *Nkx2.1*. *Nkx2.1*+ NSCs cultured from the ventral V-SVZ of postnatal mice continued to stably express *Nkx2.1* and generated neurons *in vitro*. Prolonged inhibition of the Shh pathway by cyclopamine and vismodegib had no effect on *Nkx2.1* expression. Chromatin analysis revealed that MLL1 was significantly enriched at the *Nkx2.1* promoter as well as at a putative enhancer. Acute deletion of *Mll1* from ventral cultures decreased the expression of *Nkx2.1* in a Shh-independent manner. These data support a model in which *Mll1* functions as part of a cellular memory system to

"remember" the positional identity of specific populations of NSCs over substantial periods of time.

INTRODUCTION

The adult V-SVZ is a neurogenic zone in the adult forebrain that produces olfactory bulb (OB) interneurons throughout life (Lim and Alvarez-Buylla, 2014; Zhao et al., 2008). V-SVZ precursors generate distinct subtypes of OB interneurons depending on their location along the dorsoventral and rostrocaudal axes of the lateral ventricle (Merkle et al., 2007). While some studies have suggested that the developmental fate of V-SZV precursors is cell-intrinsic (Merkle et al., 2007), others have found that extracellular signals, such as Shh signaling, might influence fate decisions (Ihrie et al., 2011). Like the germinal zone of the embryonic telencephalon, the V-SVZ is divided into distinct domains by transcription factor expression (Lopez-Juarez et al., 2013; Merkle et al., 2014) and this is thought to underlie regional differences in OB interneuron fate specification (Lledo et al., 2008). Moreover, many transcription factors expressed in the embryonic germinal zone are also expressed in a similar location of the adult V-SVZ. For instance, *Nkx2.1* is expressed in the ventral germinal zone of both the embryonic and adult brain. I have previously shown that embryonic day 12.5 (E12.5) *Nkx2.1*+ embryonic precursors give rise to NSCs in the ventral V-SVZ (Figure 2.5). Interestingly, I find that NKX2.1 expression is maintained throughout development, as virtually every *Nkx2.1*+ cell labeled at E12.5 continues to express NKX2.1 in adulthood. Over the course of development, the brains grows substantially in size and changes in morphology; how ventral neural precursors maintain *Nkx2.1* throughout embryogenesis and into adulthood is not known.

While it is unclear how *Nkx2.1* expression is maintained over the course of development, it has been shown that the induction of *Nkx2.1* in the ventral neural plate requires Shh signaling (Dale et al., 1997; Fuccillo et al., 2004; Shimamura and Rubenstein, 1997). Shh signaling is essential for proper brain development and drives neural precursor proliferation (Britto et al.,

2002; Rowitch et al., 1999; Wechsler-Reya and Scott, 1999) as well as the ventral fate specification of neural precursors throughout the central nervous system (Chiang et al., 1996; Echelard et al., 1993; Ericson et al., 1995). The hedgehog signaling pathway is highly conserved across development (Platt et al., 1997) and involves the obligate transmembrane receptors *Ptch1* and *Smo* (Alcedo et al., 1996; Stone et al., 1996). The conditional deletion of Shh pathway genes shortly after the establishment of *Nkx2.1* expression results in the downregulation of *Nkx2.1,* suggesting that *Nkx2.1* expression remains dependent on Shh activity following its initial establishment (Machold et al., 2003; Xu et al., 2005; Yu et al., 2009). Similarly, the addition of cyclopamine, an Smo receptor antagonist, to E12.5 slice cultures results in the substantial loss of *Nkx2.1* expression throughout the MGE (Gulacsi and Anderson, 2006), suggesting that *Nkx2.1*-expressing progenitors, which I have shown give rise to NKX2.1+ V-SVZ precursors, remain sensitive to Shh signaling during this period.

In the adult V-SVZ, Shh signaling continues to play an important role in the regulation of neural precursor proliferation and self-renewal (Balordi and Fishell, 2007a, b; Han et al., 2008; Ihrie et al., 2011; Machold et al., 2003), but its role in cell-fate specification is not fully understood. The extracellular environment of the ventral V-SVZ differs from that dorsally due to the close proximity of Shh-producing neurons located nearby in the bed nucleus of the stria terminalis (Ihrie et al., 2011). Deletion of Shh in adulthood appears to alter the fate of ventral V-SVZ NSCs (Ihrie et al., 2011), indicating that Shh signaling might regulate the fate of V-SVZ precursors. However, Merkle and colleagues find that ventral V-SVZ NSCs retain their developmental fates following heterotopic transplant to the dorsal V-SVZ (Merkle et al., 2007), indicating that the developmental fate of V-SVZ precursors is cell autonomous and not dependent on the extracellular environment. It is currently unclear what role Shh plays in these two seemingly divergent results.

Chromatin-based epigenetic modifiers such as trithorax and polycomb group (trxG and PcG) members are required to maintain the regional expression of homeotic transcription

factors during development in a stable and heritable manner (Lanzuolo and Orlando, 2012; Steffen and Ringrose, 2014). In the developing axial skeleton, deletion of the trxG member *Mll1* does not affect the establishment of *Hox* gene expression, but rather *Mll1* is required to maintain this expression (Gould, 1997; Schuettengruber et al., 2011; Yu et al., 1995). While canonical *Hox* gene expression ends in the hindbrain (Tumpel et al., 2009), previous studies have shown that *Mll1* is required to maintain a transcriptional program required for V-SVZ neurogenesis suggesting that trxG and PcG members might serve as important mediators of gene regulation in the forebrain (Lim et al., 2009; Potts et al., 2014). While trxG and PcG members have been studied in the context of neurogenesis (Hwang et al., 2014; Park et al., 2014; Zhang et al., 2014), little is known about their role in maintaining regional transcription factor expression.

MATERIALS AND METHODS

Animal Husbandry and Procedures

All experiments were performed with mice of either sex and in accordance to protocols approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco. C57BL/6 (wild type) mice were obtained from Jackson Laboratories. Mice homozygous for Ai14 (Madisen et al., 2010) were mated to mice with the *Nkx2-1*tm1.1(cre/ERT2)Zjh allele (Taniguchi et al., 2011). Mice homozygous for the *Mll1fl/fl* allele were provided by Dr. Patricia Ernst. For the purpose of embryonic *Nkx2.1*-CreER labeling, timed pregnant dams with *Nkx2.1*-CreER; Ai14 embryos were given one dose of tamoxifen (5mg per 30g body weight) by oral gavage at E17.5. Embryonic day 0.5 (E0.5) was estimated to be 12:00 pm on the day that vaginal plugs were observed.

Cell Culture

300 μm coronal sections were derived from P7 brains using a VT1000S vibratome (Leica).

Ventral and dorsal V-SVZ was then dissected in Lebovitz-15 (UCSF cell culture facility) media with methods as in Merkle et al. (2007). Dissected tissue was incubated with 0.25% trypsin (UCSF cell culture facility) for 20 minutes and mechanically triturated. Cells were then cultured as a monolayer on tissue culture-treated plastic plates in proliferation media containing DMEM/F-12 + Glutamax (Invitrogen), 5% FBS (Hyclone), N2 (Invitrogen), 35 ug/ml bovine pituitary extract (Invitrogen) 20 ng/ml human FGF (Peptrotech) and 20 ng/ml human EGF (Peptrotech) as previously described (Scheffler et al., 2005). For fate-tracing experiments, 4-hydroxytamoxifen (4-OHT) (Sigma) was added to cell culture media at 100 nM for 48 hours. For *Mll1* deletion experiments, 4-OHT was added to cell culture media at 100 nM for 96 hours. To induce differentiation, I removed the proliferation media, rinsed once with PBS, and then added differentiation media containing DMEM/F-12 + Glutamax, 2% FBS, N2, 35 ug/ml bovine pituitary extract. In preparation of imaging, cultures were grown on Nunc Lab-tek II Chamber Slides (Fisher). Cultures were fixed with 4% paraformaldehyde (PFA, Sigma) for 20 min at room temperature. EdU (Life Technologies) was administered at 1nM for 1 hr. For Shh-pathway experiments, Cyclopamine (Selleckchem,5uM), Vismodegib (Selleckchem, 100nM), or SAG (EMP-Millipore, 30nM).

Immunohistochemistry

Embryonic brains were fixed in 4% PFA. For cryoprotection, I equilibrated brains in PBS with 30% sucrose. After equilibration, brains were then embedded in OCT (Tissue-Tek). 12 μm cryosections were cut at -20°C in a Microm HM 525 cryostat (Thermo Scientific) and stored at -80°C. Immunohistochemistry (IHC) was performed with protocols as previously described (Hwang et al., 2014). Primary antibodies used in this study include: CalB (mouse, 1:1000, Swant), dsRed (rabbit, 1:1000, Living Colors), Nestin (chicken, 1:500, Aves), Nkx2.1 (rabbit, 1:400, Santa Cruz), Nkx2.1 (mouse IgG1, 1:250, Leica), RFP (rat, 1:1000, Chromotek), Tuj1

(mouse, 1:1000, Covance). Alexfluor conjugated secondary antibodies (Life Technologies) were used at 1:500. Nuclear counterstain DAPI (Sigma) was applied at 1:100000.

RNA-seq *Analysis*

Experiments were performed in triplicate. RNA was extracted using TRIzol (QIAGEN) and purified/DNAse-treated on RNesay columns (QIAGEN). Strand-specific, poly(A) selected cDNA libraries were generated using TruSeq Stranded mRNA kit (illumina) according to the manufacturer's protocol. Library validation and normalization were performed using RT-PCR and Quant-iT PicoGreen (Invitrogen). Cluster generation and high-throughput sequencing were performed on a HiSeq 2500 (Illumina), using the single-end 50 bp protocol. Reads were aligned to the mouse genome mm9, using Tophat v2.0.10 with the following arguments: -p 6 --library-type fr-firststrand. Differential expression was assessed using Cuffdiff v2.1.1 with the following arguments: -b genome.fa -u --library-type fr-firststrand. A transcriptome index that includes all UCSC genes was used for Tophat (Trapnell et al., 2009) and Cufflinks (Trapnell et al., 2012).

qRT-PCR

RNA was extracted using TRIzol as described above. cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche). qRT-PCR was performed on a Roche Light Cycler 480 II.

Primers:

RPLP0-F: CCGATCTGCAGACACACACT

RPLP0-R: ACCCTGAAGTGCTCGACATC

Gsx2-F: CATCATCAAGGACTCCTCACG G

Gsx2-R: GACATCACCAACGGGGACG

Gsx1-F: TCTGTGGACAGCAGCTCGAA

Gsx1-R: ACATGTTGGAGGCGAACTCT

Nkx6-2-F: AAGGTGTGGTTCCAGAATCGGCG

Nkx6-2-R: ACCCACCTTCAGCTTCTCGGCA

Gli1-F: TAGGGTCTCGGGGTCTCAAACTGC

Gli1-R: ACCTGCGGCTGACTGTGTAAGCA

Nkx2.1-F: GGCATGGCACAGTTTTGGAGC C

Nkx2.1-R: TCACTTTGCTGGCAGGGTGCA T

Emx1-F: TCCGAGACGCAGGTGAAGGTG T

Emx1-R: TTCTGCTCAGACTCCGGCCCT T

Ptch1-F: CGTCCACGTGGCTTTGGCCTT

Ptch1-R: CAGCACCGTCCAGAACGGGAG

Shh-F: AAAGCAGAGAACTCCGTGGCGG

Shh-R: TCCGGGACGTAAGTCCTTCACCA

Smo-F: TCTGGTCCGGCCTCCGGAAT

Smo-R: CGGCTGGGCAACTCCACTCG

Chromatin immunoprecipitation

qChIP and ChIP-seq were performed using standard methods using the antibodies: MLL1 (Mouse, 1ug, Bethyl), H3K4me3 (Rabbit, 2ul, Active Motif), and IgG (Mouse, 1ug, Santa Cruz). For ChIP-seq analysis, reads were mapped to the mm9 genome with Bowtie2 (Langmead and Salzberg, 2012) and peaks were called with MACS (Zhang et al., 2008)

Imaging and quantification

Imaging was performed using either a DMI4000 B epifluorescent microscope with attached DFC345 FX camera or a TCS SP5 X confocal microscope (Leica). Image processing including cropping and pseudo-coloring was performed with Fiji (Schindelin et al., 2012) and Photoshop CC (Adobe). Quantification of NKX2.1+ cells per unit area was performed using Fiji. Student's t-tests were performed using Prism 6 (Graph Pad).

RESULTS

Ventral and Dorsal V-SVZ NSCs retain regional identity when cultured *in vitro*

In order to determine how ventral V-SVZ NSCs maintain *Nkx2.1* expression throughout life, I utilized a monolayer culture system that enriches for V-SVZ NSCs (Scheffler et al., 2005). Under proliferative conditions, NSC monolayer cultures can be passaged for an extended period of time, allowing me to greatly expand the pool of V-SVZ NSCs. From 300 μm coronal sections of P7 brains, I microdissected both the dorsolateral and ventral V-SVZ, dissociated the tissue, and established NSC monolayer cultures (Figure 3.1A). After 5 passages (~10 days *in vitro*), both ventral and dorsolateral cultures expressed Nestin (Figure 3.1B), a marker of V-SVZ NSC identity. To determine whether regional V-SVZ cultures remained neurogenic, I differentiated the cultures by switching to a low-growth factor "differentiation" medium. After 4 days in differentiation medium, I observed that both dorsolateral and ventral cultures gave rise to Tuj1+ neuroblasts (Figure 3.1C). Previous fate-tracing studies have found that CalB+ OB interneurons are born from the ventral, but not dorsal V-SVZ (Merkle et al., 2007). Consistent with these *in vivo* data, I observed CalB+/Tuj1+ cells in ventral, but not dorsal, cultures (Figure 3.1C). This result suggested that regional differences in V-SVZ NSC identity are maintained *in vitro* when cultured outside of the V-SVZ niche.

Next, I investigated whether regional transcription factor expression was maintained by V-SVZ NSCs cultured *in vitro*. In the adult brain, *Gsx1* and *Gsx2* expression form complimentary domains along the dorsoventral axis of the V-SVZ, with *Gsx1* expressed ventrally and *Gsx2* expressed in the dorsolateral corner (Lopez-Juarez et al., 2013). I found that this pattern was maintained *in vitro*, with *Gsx2* expression restricted entirely to dorsal cultures (Figure 3.1D) and *Gsx1* significantly enriched ventrally (Figure 3.1E). Dorsolateral cultures were also significantly enriched for Emx1 *(*Figure 3.1D*)*, a transcription factor expressed in the embryonic pallium which later gives rise to NSCs in the dorsal V-SVZ (Kohwi et al., 2007; Young et al., 2007). The

Nkx homeobox transcription factors *Nkx2.1* and *Nkx6.2* are expressed by ventral V-SVZ precursors (Merkle et al., 2014). I found that ventral cultures were enriched for the Nkx homeobox transcription factors *Nkx2.1* and *Nkx6.2* (Figure 3.1D). *Nkx2.1* expression was exclusive to ventral cultures as dorsal cultures lacked detectable levels of *Nkx2.1* mRNA (Figure 3.1D). ICC analysis of ventral cultures revealed that 35% of cells expressed NKX2.1 protein (3436 of 9886 cells, Figure 3.1*F*). In contrast, NSC cultures derived from dorsal P7 V-SVZ did not express NKX2.1 protein (data not shown). Many NKX2.1+ cells expressed Nestin (Figure 3.1F), a V-SVZ NSC marker. Thus, regional differences in gene expression and neuronal fate specification present in the adult V-SVZ are maintained *in vitro*.

Postnatal expression of *Nkx2.1* in V-SVZ NSCs is stable and heritable

My previous fate mapping studies revealed that embryonic *Nkx2.1*-expressing neural precursors persist throughout development and give rise to NKX2.1+ NSCs in the V-SVZ (Figure 2.5). Over 99% of E12.5-labeled cells residing in the V-SVZ continued to express NKX2.1 protein at P60, suggesting that *Nkx2.1* expression is robust and maintained over a period of rapid tissue growth and cell division. In order to determine whether *Nkx2.1* expression was maintained heritably in proliferating NSCs, I employed a genetic fate-tracing strategy with the *Nkx2.1*-CreER allele, in which the CreER expression cassette is inserted into the endogenous *Nkx2.1* locus (Taniguchi et al., 2011). To follow the fate of cells that had undergone Cre-mediated recombination, I used the Ai14 transgene, which expresses tdTomato after excision of a “floxed-stop” cassette (Madisen et al., 2010). Following administration of 4-hydroxytamoxifen (4-OHT), I detected tdTomato+ cells in ventral V-SVZ cultures derived from *Nkx2.1*-CreER; Ai14 mice (Figure 3.2A). Consistent with the absence of NKX2.1 immunoreactive cells in dorsal V-SVZ cultures, I did not observe tdTomato+ cells in 4-OHT treated dorsal cultures derived from *Nkx2.1*-CreER; Ai14 mice (Figure 3.2A). Following 15 days (d) of serial passage under proliferative conditions, every tdTomato+ cell expressed NKX2.1 protein (100%, 605 of 605 cells, Figure 3.2A). To determine whether *Nkx2.1*+ NSCs can give

rise to neurons, I labeled *Nkx2.1*-expressing cells under proliferative conditions and then switched to differentiation medium to promote neurogenesis. After 4d in differentiation medium, I observed Tuj1+/tdTomato+ cells with a neuronal morphology in ventral V-SVZ cultures (Figure 3.2B). Thus, *Nkx2.1*+ NSCs derived from the postnatal V-SVZ maintain expression of *Nkx2.1* in proliferation conditions and can generate neurons *in vitro.*

Postnatal Expression of *Nkx2.1* does not require Shh signaling

Shh signaling is required for the expression of many transcription factors in the ventral embryonic forebrain including *Nkx2.1* and *Nkx6.2* (Fuccillo et al., 2004; Machold et al., 2003; Xu et al., 2005; Yu et al., 2009). My previous fate-tracing analysis revealed that adult NKX2.1+ V-SVZ NSCs are derived from *Nkx2.1*-expressing embryonic progenitors labeled at E12.5 (Figure 2.5), a time at which a loss of Shh signaling results in decreased *Nkx2.1* expression (Gulacsi and Anderson, 2006). Previous studies have found that Shh signaling is enriched in the ventral V-SVZ and the deletion of *Shh* in adulthood results in the decreased production of ventral-type OB interneurons (Ihrie et al., 2011). These data suggest that Shh signaling might be required to maintain the ventral identity of V-SVZ NSCs.

To determine whether the maintenance of *Nkx2.1* expression in the postnatal V-SVZ NSCs requires Shh signaling, I assessed regional cultures for the expression of canonical Shh pathway genes. Consistent with previous *in vivo* studies (Angot et al., 2008; Ihrie et al., 2011), I found that both dorsal and ventral V-SVZ NSCs expressed *Ptch1* and *Smo*, the obligate transmembrane receptors required for Shh signal transduction (Alcedo et al., 1996; Stone et al., 1996) (Figure 3.3A). However, *Shh* and *Gli1* mRNA were detected exclusively in ventral cultures (Figure 3.3A), indicating that Shh signaling persists *in vitro* and is restricted to ventral cultures. To determine whether *Nkx2.1* expression in ventral V-SVZ NSCs required Shh signaling, I blocked Shh signal transduction by using cyclopamine and vismodegib, pharmacological antagonists of the Shh pathway. I treated cultures for 6 passages (10 days *in vitro*) and then

quantified the proportion of cells that expressed NKX2.1 protein (Figure 3.3B). Both cyclopamine and vismodegib treatment significantly reduced *Gli1* and *Ptch1* expression, indicating that the Shh pathway was effectively blocked (Figure 3.3C). However, Shh-pathway antagonism had no effect on *Nkx2.1* mRNA levels (Figure 3.3C) or on the proportion of NKX2.1+ cells (Figures 3.3D,E). Addition of the Shh pathway agonist SAG (smoothened agonist), had no effect on *Nkx2.1* expression (Figure 3.3C) or on the proportion of NKX2.1+ cells (Figures 3.3D,E). In contrast, *Nkx6.2* expression responded to changes in Shh signaling and was moderately increased upon Shh pathway activation and significantly decreased in cyclopamine- and vismodegib-treated cultures (Figure 3.3C). Thus, unlike embryonic precursors in the early embryonic telencephalon that require Shh signaling to maintain *Nkx2.1* expression, postnatal V-SVZ NSCs maintain *Nkx2.1* expression in a stable and heritable manner, independent of Shh signaling.

Mll1 is required to maintain *Nkx2.1* expression

PcG and *trxG* members are central members of cellular memory systems that maintain a heritable state of gene expression following an inductive signal (Lanzuolo and Orlando, 2012; Schuettengruber et al., 2011; Steffen and Ringrose, 2014). While *Nkx2.1* expression requires Shh signaling early in embryogenesis, my data suggested that postnatal *Nkx2.1* expression is maintained in a stable and heritable manner (Figure 3.2) independent of Shh signaling (Figure 3.3). I hypothesized that *Nkx2.1* expression was being maintained by a cellular memory system.

Chromatin immunoprecipitation (ChIP) analysis of the *Nkx2.1* locus revealed that the TrxG member MLL1 was enriched at the promoter and putative enhancer of *Nkx2.1* (Rada-Iglesias et al., 2011) in ventral but not dorsal cultures (Figure 3.4A). Trimethylation of lysine 4 in histone H3 (H3K4me) is a chromatin modification associated with active gene expression (Kouzarides, 2007; Schuettengruber et al., 2007). Consistent with the ventral expression of *Nkx2.1*, ChIP-seq analysis revealed that the *Nkx2.1* locus in ventral but not dorsal cultures was

enriched for H3K4me3 (Figure 3.4B). In contrast, the zinc-finger transcription factor *Sp8*, which is expressed in both the ventral and dorsal V-SVZ (Waclaw et al., 2006) had H3K4me3 enrichment in both dorsal and ventral cultures (Figure 3.4C).

To determine whether MLL1 is required to maintain *Nkx2.1* expression, I used the *UBC-CreER* transgene (Ruzankina et al., 2007) to drive the deletion of conditional "floxed" *Mll1* alleles (*Mll1*$^{f/f}$). Cre-mediated recombination of the conditional *Mll1* allele results in the deletion of exons 3 and 4 which encode the nuclear targeting sequence and result in a null allele (Jude et al., 2007). I derived ventral V-SVZ cultures from *UBC-CreER Mll1*$^{f/f}$ animals and *UBC-CreER:Mll1*$^{f/+}$ littermates (hereafter referred to as control). After expanding cultures for 3 passages, I added 4-Hydroxytamoxifen (4-OHT) to the culture medium for 4 days in order to drive Cre-mediated recombination (Figure 3.5A). RNA-seq analysis confirmed the expected loss of exons 3 and 4 in Mll1$^{f/f}$ cultures after 4-OHT treatment as compared to control (Figure 3.5B). Furthermore, ICC analysis demonstrated the absence of MLL1 immunoreactivity in the nuclei of 4-OHT-treated *Mll1f/f* cells (Figure 3.5C). Differential expression analysis by RNA-seq revealed that 1427 genes were upregulated and 799 were downregulated at an FDR < 0.05 following *Mll1* deletion (Figures 3.5D). *Nkx2.1* was the second-most significantly downregulated gene (Figures 3.5D,E). The GO terms "Forebrain Development" and "Neuron Differentiation" were the two most significantly enriched categories (p=2.4E-6 and p=3.0E-6) amongst down-regulated genes. These categories included the genes *Nkx2.1*, *Gsx1, Olig2, Olig1,* and *Ascl1* (Figures 3.5D-E), which are involved in embryonic forebrain development (Lu et al., 2000; Pei et al., 2011; Sussel et al., 1999; Toresson and Campbell, 2001; Wang et al., 2009). I used qRT-PCR analysis to confirm that the expression of *Nkx2.1, Gsx1, Olig2, and Olig1* was decreased in *Mll1f/f* cultures (Figure 3.5F).

GO term analysis revealed that the "Hedgehog Signaling Pathway" was significantly upregulated following *Mll1* deletion (p=8.3E-2). Both *Ptch1* and *Gli1* were significantly upregulated (Figures 3.*5C,D*) suggesting that Shh signaling was not lost secondary to *Mll1*

deletion. Interestingly, the ventrally enriched gene *Nkx6.2*, which I previously found to be sensitive to changes in Shh signaling (Figure 3.3C) was significantly upregregulated, indicating that it did not require *Mll1* for expression. Consistent with decreased *Nkx2.1* mRNA levels observed by RNA-seq (Figures 3.5D-F), I found that *Mll1*-deleted cultures had a significant reduction in the proportion of cells that expressed NKX2.1 protein (Figures 3.6A,B).

To determine whether differences in cell proliferation might account for the loss of NKX2.1+ cells following *Mll1* deletion, I administered the thymidine analog 5-Ethynyl-2´-deoxyuridine (EdU) to Mll1$^{f/f}$ and control cultures for 1 hour prior to analysis. ICC analysis revealed that the proportion of NKX2.1+ cells labeled with EdU was the same in both *Mll1*$^{f/f}$ and control cultures (Figure 3.6B), suggesting that NKX2.1+ cells did not divide more slowly following *Mll1* deletion. Furthermore, *Mll1* deletion did not have an effect on the proportion of NKX2.1- cells that incorporated EdU (Figure 3.6C). To determine whether the decrease in NKX2.1+ cells was due to cell death, I performed immunocytochemistry for cleaved-caspase3, a marker of apoptosis, and found that cleaved-caspase3 was not altered in *Mll1*$^{f/f}$ cultures. These data are consistent with previous studies of *Mll1* deletion in V-SVZ NSCs (Lim et al., 2009) and suggest that the loss of NKX2.1+ cells is not due to changes in cell death or proliferation. Consistent with decreased *Nkx2.1* expression, chromatin analysis revealed a decrease in H3K4me3 at the *Nkx2.1* promoter following *Mll1* deletion (Figure 3.6D). Taken together, these data suggest a model in which *Mll1* maintains the active transcriptional state of *Nkx2.1* in the postnatal V-SVZ (Figure 3.6E).

Nkx2.1 expression does not require autoregulation

Autoregulation is an important mechanism underlying the maintenance of *Hox* gene expression during embryogenesis (Lou et al., 1995; Packer et al., 1998). The *Nkx2.1* regulatory region contains evolutionarily conserved NKX2.1-binding sites and *in vitro* studies revealed that NKX2.1 can bind and positively regulate transcription from its own promoter (Das et al., 2011;

Oguchi and Kimura, 1998). To determine whether autoregulation is required to maintain *Nkx2.1* expression in the ventral telencephalon (Figure 3.7A), I studied the brains of *Nkx2.1*-CreER:*Ai14* mice that carry a "knock-in/knock-out" *Nkx2.1*-CreER allele that closely recapitulates endogenous *Nkx2.1* expression (Taniguchi et al., 2011). While *Nkx2.1*-CreER heterozygotes (*Nkx2.1*-CreER/+) carry one wildtype *Nkx2.1* allele, Nkx2.1-CreER homozygotes (*Nkx2.1*-CreER/CreER) lack a functional *Nkx2.1* allele and instead carry two mutant *Nkx2.1* alleles that encode CreER (Figure 3.7C). Transcriptional activity from the *Nkx2.1* locus can be detected by tamoxifen-induced, Cre-mediated recombination of the *Ai14* transgene (Figure 3.7C). In order to circumvent neonatal lethality observed in *Nkx2.1*-null mice (Kimura et al., 1996), I administered tamoxifen at E17.5 and collected embryos at E18.5 (Figure 3.7B). Consistent with the expression pattern observed by Taniguchi et al., NKX2.1 and tdTomato expression were detected ventrally along the lateral walls of the lateral ventricle (Figure 3.7D). While *Nkx2.1*-CreER/CreER mice lacked NKX2.1 protein, tdTomato was expressed in the ventral subpallium (Figure 3.7D) suggesting that transcription of the *Nkx2.1* locus does not require NKX2.1 protein.

DISCUSSION

My results indicate that *Nkx2.1* expression is maintained in ventral V-SVZ NSCs in a stable and heritable manner independent of Shh signaling. Chromatin analysis revealed that the trxG chromatin modifier MLL1 binds *Nkx2.1* regulatory elements and is required to maintain the postnatal expression of *Nkx2.1*. These results indicate that *Nkx2.1* expression is maintained in the postnatal V-SVZ by a cellular memory system that requires *Mll1*.

The differences I observed in the postnatal regulation of *Nkx2.1* and *Nkx6.2* expression highlight two different ways in which regional gene expression is maintained in the postnatal V-

SVZ (summarized in Figure 3.6E). In contrast to *Nkx2.1*, I found that postnatal *Nkx6.2* expression is dependent on Shh signaling (Figure 3.3C). Changes in Shh signaling might therefore affect the production/specification of OB interneurons born from *Nkx6.2*+ V-SVZ precursors and not those born from *Nkx2.1*+ precursors. This may contribute to the phenotype reported by Ihrie and colleagues, who observed that conditional deletion of *Shh* in adulthood results in the decreased production of deep GC OB interneurons (Ihrie et al., 2011). While it is currently unknown whether *Nkx6.2* is required to specify the developmental fate of V-SVZ-born OB interneurons, I hypothesize that this population of precursors would be sensitive to changes in Shh signaling whereas the *Nkx2.*1+ populations of V-SVZ precursors would not.

My data indicate that Shh is expressed *in vitro* within ventrally-derived V-SVZ NSC cultures (Figure 3.3A). In adulthood, *Shh*+ cells are located in the medial septum, in ventral forebrain, and at low numbers in the cortex (Ihrie et al., 2011; Traiffort et al., 1999). Histological analysis reveals that the majority of Shh+ cells in the adult forebrain express the neuronal marker NeuN and have neuronal morphologies (Garcia et al., 2010). However, under proliferative conditions, Tuj1+ and NeuN+ cells are not observed in culture (data not shown and (Scheffler et al., 2005). This suggests that a population of EGF/FGF responsive cells in the ventral V-SVZ produce Shh. Further analysis of specific V-SVZ precursor populations will be helpful in determining the identity of *Shh*-expressing cells.

The observed upregulation of the Shh pathway following *Mll1* deletion (Figures 3.5D-F) indicates that *Mll1* is not required for transduction of Shh signaling. Thus, decreased *Nkx2.1* expression following *Mll1* deletion (Figure 3.5D-F) is not secondary to a disruption in the Shh pathway. Furthermore, the upregulation of Shh pathway following *Mll1* deletion indicates that *Mll1* might serve to attenuate Shh pathway activity. Similar results have been observed in *Brg1* TrxG mutants. Embryonic mice lacking *Brg1*, an ATP-dependent chromatin remodeling factor, have increased Shh signaling throughout the central nervous system and ectopic expression of *Nkx6.2* in the telencephalon (Zhan et al., 2011). While Zhan et al. find that *Brg1* represses Shh

target genes through *Gli3*, it is currently unclear how *Mll1* deletion results in increased Shh signaling within the postnatal V-SVZ culture system.

Genetic labeling experiments indicate that NKX2.1+ NSCs in the adult V-SVZ derive from *Nkx2.1*-expressing embryonic precursors labeled at the time when Nkx2.1 expression is dependent on Shh signaling (Figure 2.5). It is therefore possible that *Nkx2.1* expression in neural precursors transitions from requiring Shh signaling to requiring *Mll1* at some point in development (Figure 3.6E). Consistent with this notion, blockade of the Shh signaling pathway early in development has a much more significant effect on *Nkx2.1* expression than inhibition later in development. Deletion of *Smo* by a *FoxG1*-Cre driver active at E9 results in the almost complete loss of *Nkx2.1* expression in the ventral telencephalon (Fuccillo et al., 2004). In contrast, the conditional deletion of *Smo* by a *Nestin*-Cre diver at E10.5 results in a less severe loss of *Nkx2.1* expression (Machold et al., 2003). However, careful analysis of E12.5 *NestinCre:Shh*$^{f/f}$ animals reveals that *Nkx2.1* loss is most severe in the dorsal MGE where *Gli1* mRNA is detected (Xu et al., 2005). Thus, changes in the sensitivity to Shh signaling vary amongst different populations of cells.

It in unclear when in development *Nkx2.1* expression requires *Mll1*. Previous studies reveal that TrxG/PcG members are not required for the establishment of *Hox* gene expression, but rather for their maintenance (Gould, 1997; Schuettengruber et al., 2011; Yu et al., 1998; Yu et al., 1995). I therefore would predict that *Mll1* is not required for the initial expression of *Nkx2.1*. Due to the previously discussed decrease in sensitivity to Shh signaling observed around E12.5, I predict that *Nkx2.1* expression would require *Mll1* around this point in development.

FIGURES

Figure 3.1

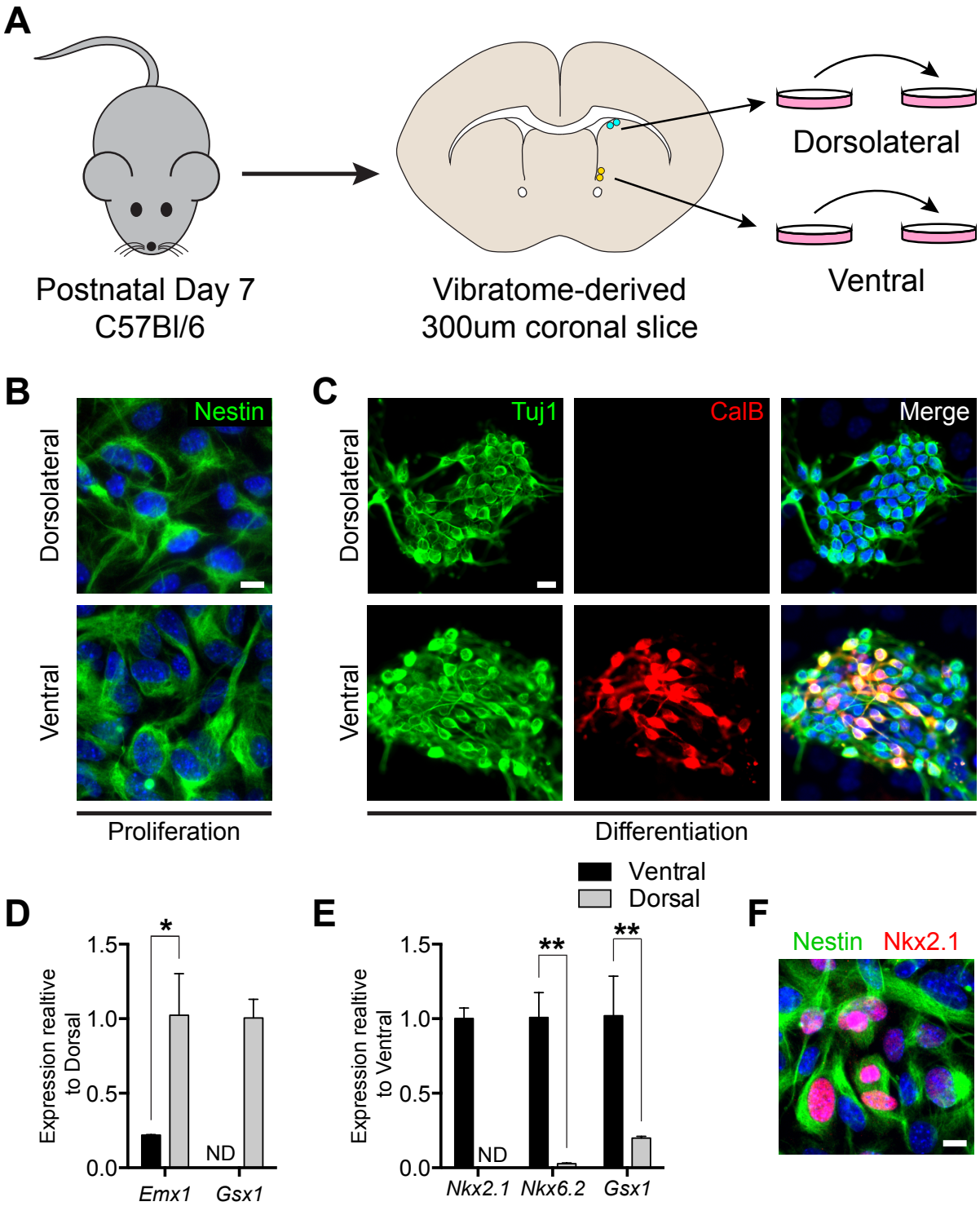

Figure 3.1 Dorsal and Ventral V-SVZ NSCs maintain regional differences *in vitro*. **A,** Regional dissection and *in vitro* culture diagram. **B,** ICC analysis of regional V-SVZ NSC cultures at passage 5 for Nestin (green) and DAPI (blue). **C,** ICC analysis of dorsal and ventral cultures following 4 days of differentiation for Tuj1 (green), CalB (red), and DAPI (blue). **D,** qRT-PCR expression analysis of dorsal and ventral cultures for genes enriched in the ventral V-SVZ. Expression relative to ventral cultures. **E,** qRT-PCR expression analysis of dorsal and ventral cultures for genes enriched in the dorsal V-SVZ. Expression relative to dorsal cultures. **F,** ICC analysis of ventral V-SVZ NSC cultures at passage 5 for Nestin (green), NKX2.1 (red), and DAPI (blue).

Scale bars: 10um.

Figure 3.2

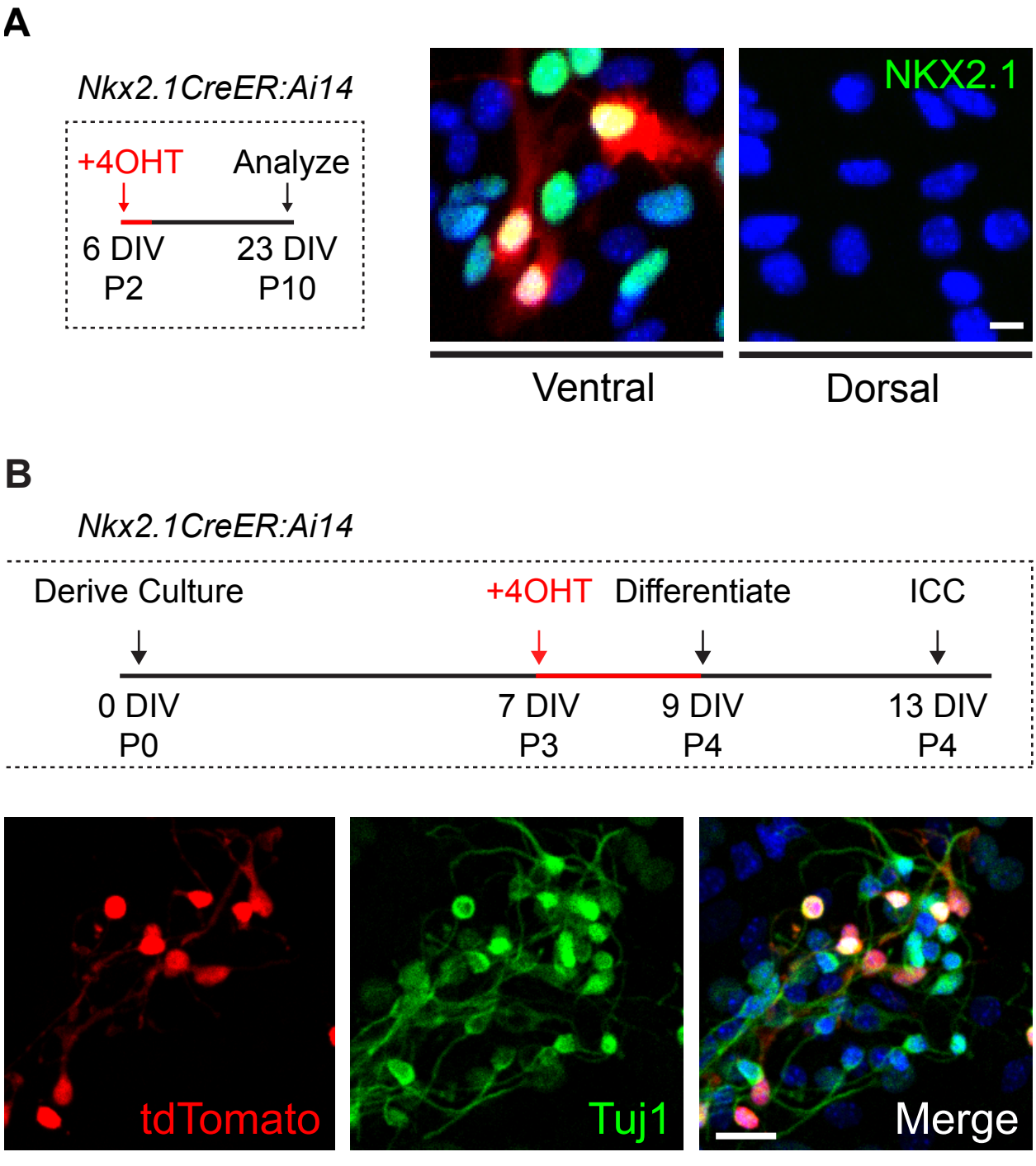
A
Nkx2.1CreER:Ai14
+4OHT
Analyze
6 DIV
P2
23 DIV
P10
NKX2.1
Ventral
Dorsal
B
Nkx2.1CreER:Ai14
Derive Culture
+4OHT
Differentiate
ICC
0 DIV
P0
7 DIV
P3
9 DIV
P4
13 DIV
P4
tdTomato
Tuj1
Merge

Figure 3.2 *Nkx2.1*+ NSCs maintain NKX2.1 expression and are neurogenic *in vitro.* **A,** Long-term *Nkx2.1*-labeling experimental design. Ventral culture derived from *Nkx2.1*-CreER:Ai14 mice, 4-OHT administered at passage 2 (6 days *in vitro*), cultures fixed and analyzed for tdTomato (red), NKX2.1 (green), and DAPI (blue) at passage 10 (23 days *in vitro*). **B,** *Nkx2.1*-labeling and differentiation experimental design. Ventral culture derived from *Nkx2.1*-CreER:Ai14 mice, 4-OHT administered at passage 3 (7 days *in vitro*), switch to differentiation media at passage 4 (9 days *in vitro*), cultures fixed and analyzed for tdTomato (red), Tuj1 (green), and DAPI (blue) at passage 5 (13 day *in* vitro) Scale bars: 10um.

Figure 3.3

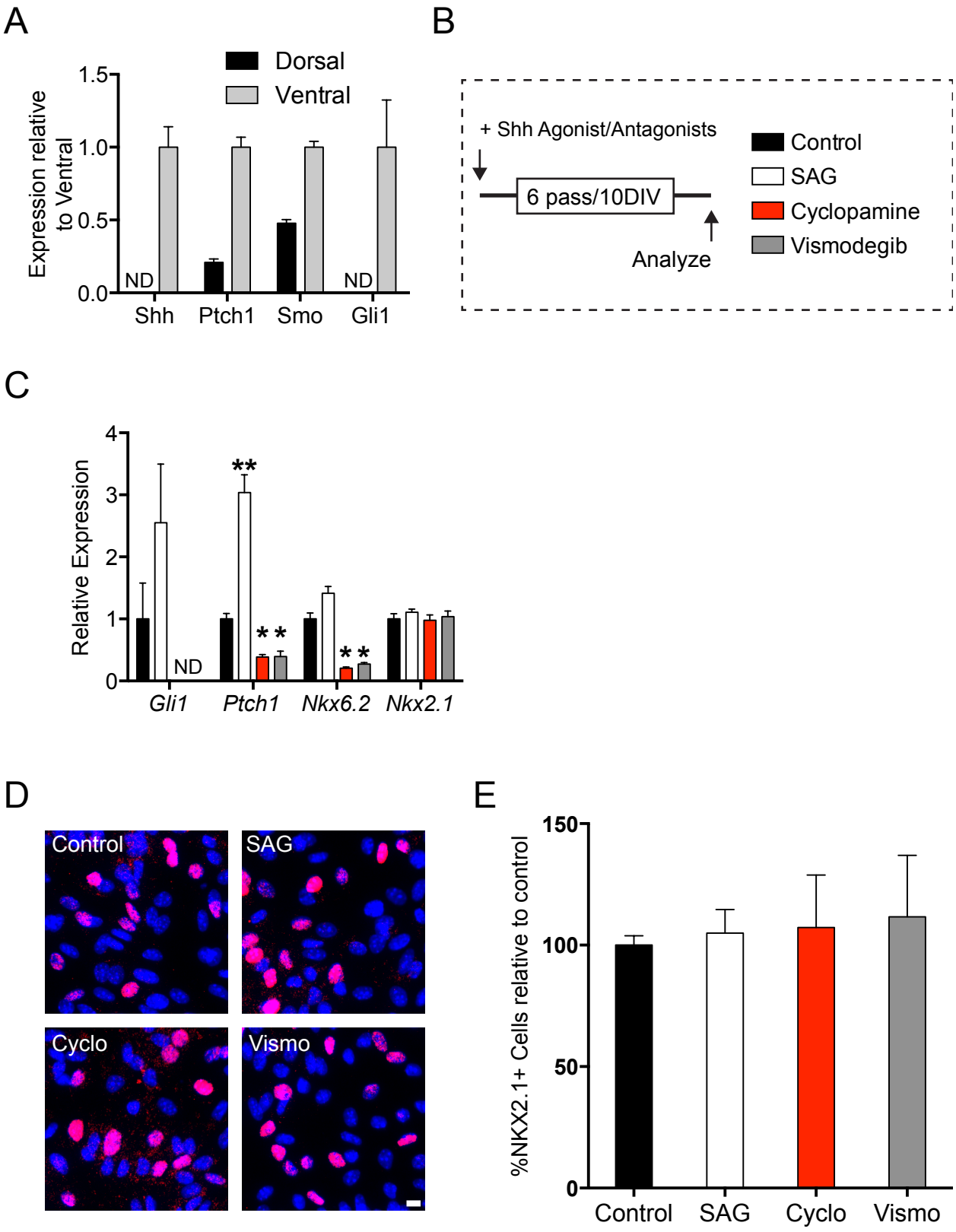
A
Dorsal
Ventral
Expression relative to Ventral
1.5
1.0
0.5
0.0
ND
ND
Shh
Ptch1
Smo
Gli1
B
+ Shh Agonist/Antagonists
6 pass/10DIV
Analyze
Control
SAG
Cyclopamine
Vismodegib
C
Relative Expression
4
3
2
1
0
**
* *
* *
ND
Gli1
Ptch1
Nkx6.2
Nkx2.1
D
Control
SAG
Cyclo
Vismo
E
%NKX2.1+ Cells relative to control
150
100
50
0
Control
SAG
Cyclo
Vismo

Figure 3.3 *Nkx2.1* expression in postnatal V-SVZ NSC cultures does not require Shh signaling. **A,** qRT-PCR analysis of canonical Shh pathway genes in ventral and dorsal cultures relative to ventral culture. ND= not detected. **B,** Long-term Shh pathway blockage diagram. **C,** ICC analysis of NKX2.1 (red) and DAPI (blue) expression after 6passages/10DIV of experimental conditions. **D,** Quantification of NKX2.1+ cells relative to control. **E,** qRT-PCR analysis of *Gli1*, *Ptch1*, *Nkx6.2*, and *Nkx2.1* expression after 6passages/10DIV of experimental conditions. Scale bars: 10um.

Figure 3.4

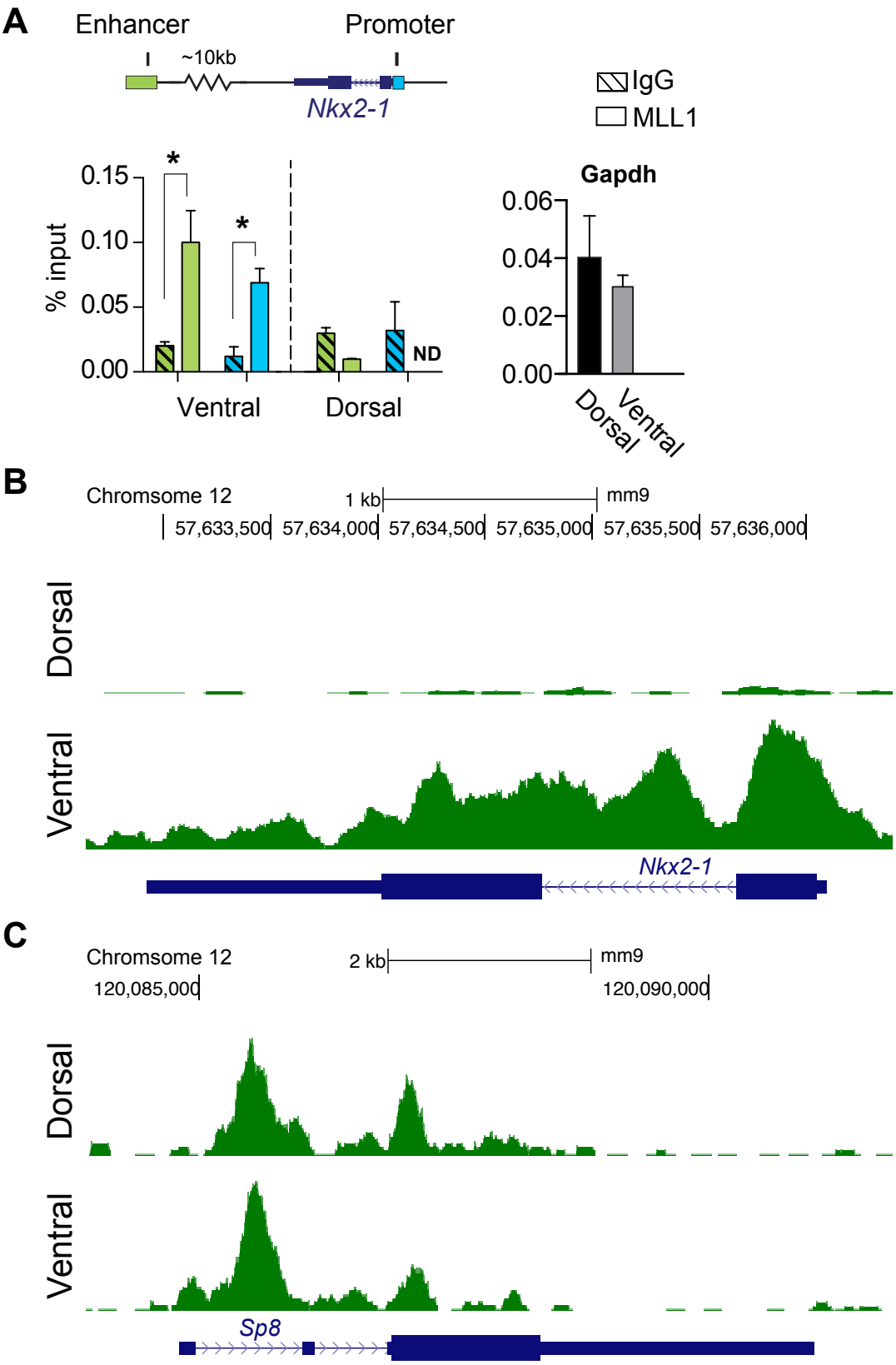
A
Enhancer
Promoter
~10kb
Nkx2-1
IgG
MLL1
% input
0.15
0.10
0.05
0.00
*
*
ND
Ventral
Dorsal
Gapdh
0.06
0.04
0.02
0.00
Dorsal
Ventral
B
Chromsome 12
1 kb
mm9
57,633,500
57,634,000
57,634,500
57,635,000
57,635,500
57,636,000
Dorsal
Ventral
Nkx2-1
C
Chromsome 12
2 kb
mm9
120,085,000
120,090,000
Dorsal
Ventral
Sp8

Figure 3.4 MLL1 binds the *Nkx2.1* locus. **A,** q-ChIP analysis of MLL1 at the *Nkx2.1* promoter and putative enhancer. *Gapdh* locus is included for positive control. **B,** ChIP-seq analysis for H3K4me3 across the *Nkx2.1* locus in ventral and dorsal cultures. **C,** ChIP-seq analysis for H3K4me3 across the *Sp8* locus in ventral and dorsal cultures.

Figure 3.5

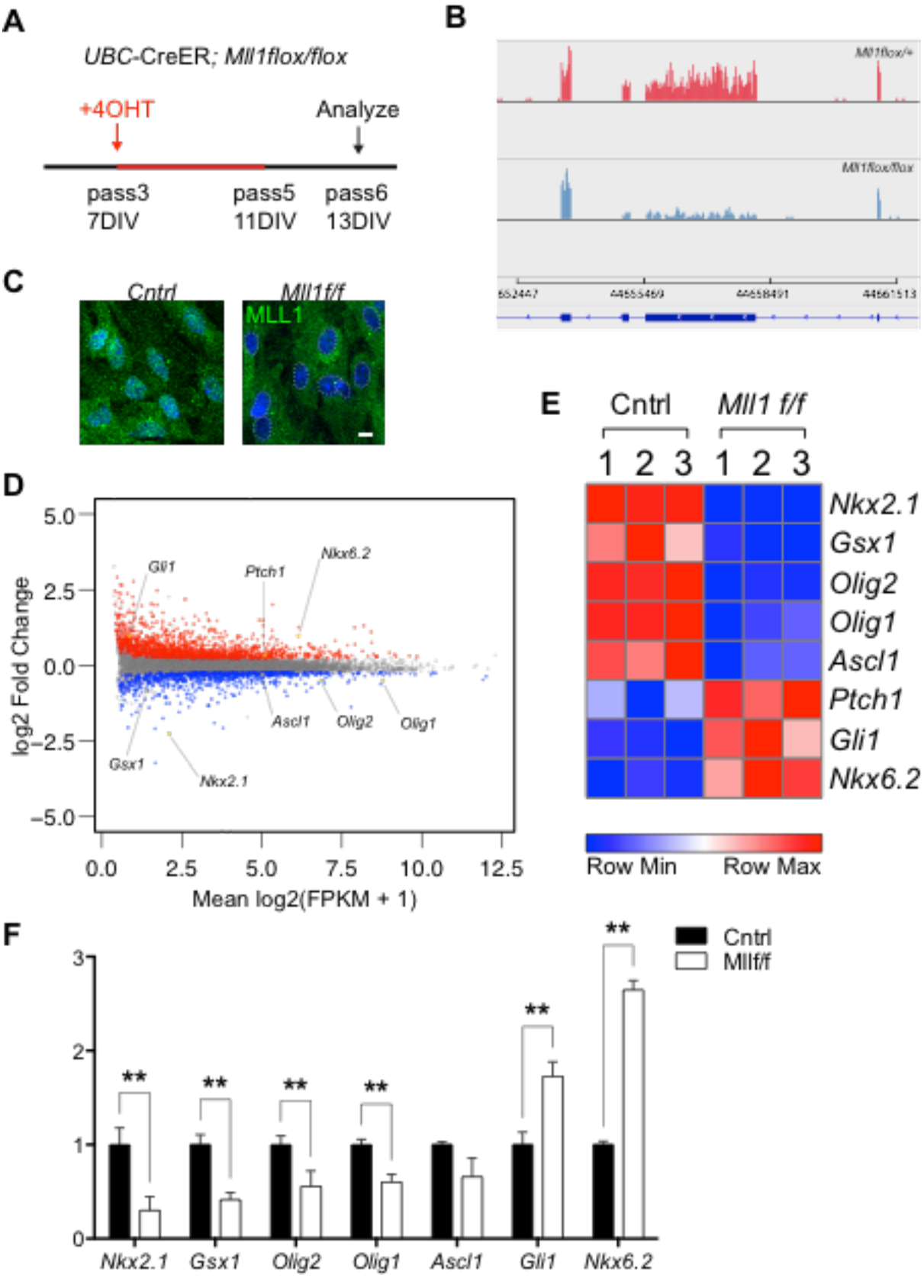
A
UBC-CreER; Mll1flox/flox
+4OHT
Analyze
pass3
7DIV
pass5
11DIV
pass6
13DIV
B
C
Cntrl
Mll1f/f
MLL1
D
log2 Fold Change
Mean log2(FPKM + 1)
Gli1
Ptch1
Nkx6.2
Ascl1
Olig2
Olig1
Gsx1
Nkx2.1
E
Cntrl
Mll1 f/f
1 2 3 1 2 3
Nkx2.1
Gsx1
Olig2
Olig1
Ascl1
Ptch1
Gli1
Nkx6.2
Row Min
Row Max
F
Cntrl
Mllf/f
Nkx2.1
Gsx1
Olig2
Olig1
Ascl1
Gli1
Nkx6.2

Figure 3.5 Acute *Mll1* deletion results in the downregulation of *Nkx2.1*. **A,** Diagram of *Mll1* deletion in ventral V-SVZ NSC culture derived from P7 *UBC-CreER:Mll1f/f* mice. **B,** RNA-seq analysis of exons 3 and 4 of *Mll1* following in *control* (blue) and *Mll1f/f* cultures (red) following administration of 4-OHT. **C,** ICC analysis of MLL1 (green) and DAPI (blue) expression in *control* and *Mll1f/f* cultures following 4-OHT administration.

D, Differential expression analysis of RNA-seq data from *control* and *Mll1f/f* cultures. Read count is depicted on X-axis. Fold change (log2) depicted on Y-axis. Dots depict individual genes. Colored dots depict significantly (FDR<0.05) upregulated (red) and downregulated (blue) genes in *Mll1f/f* cultures. **E,** Heat-map of select genes. **F,** qRT-PCR confirmation of select genes. *=p<0.05 **=p<0.01.

Figure 3.6

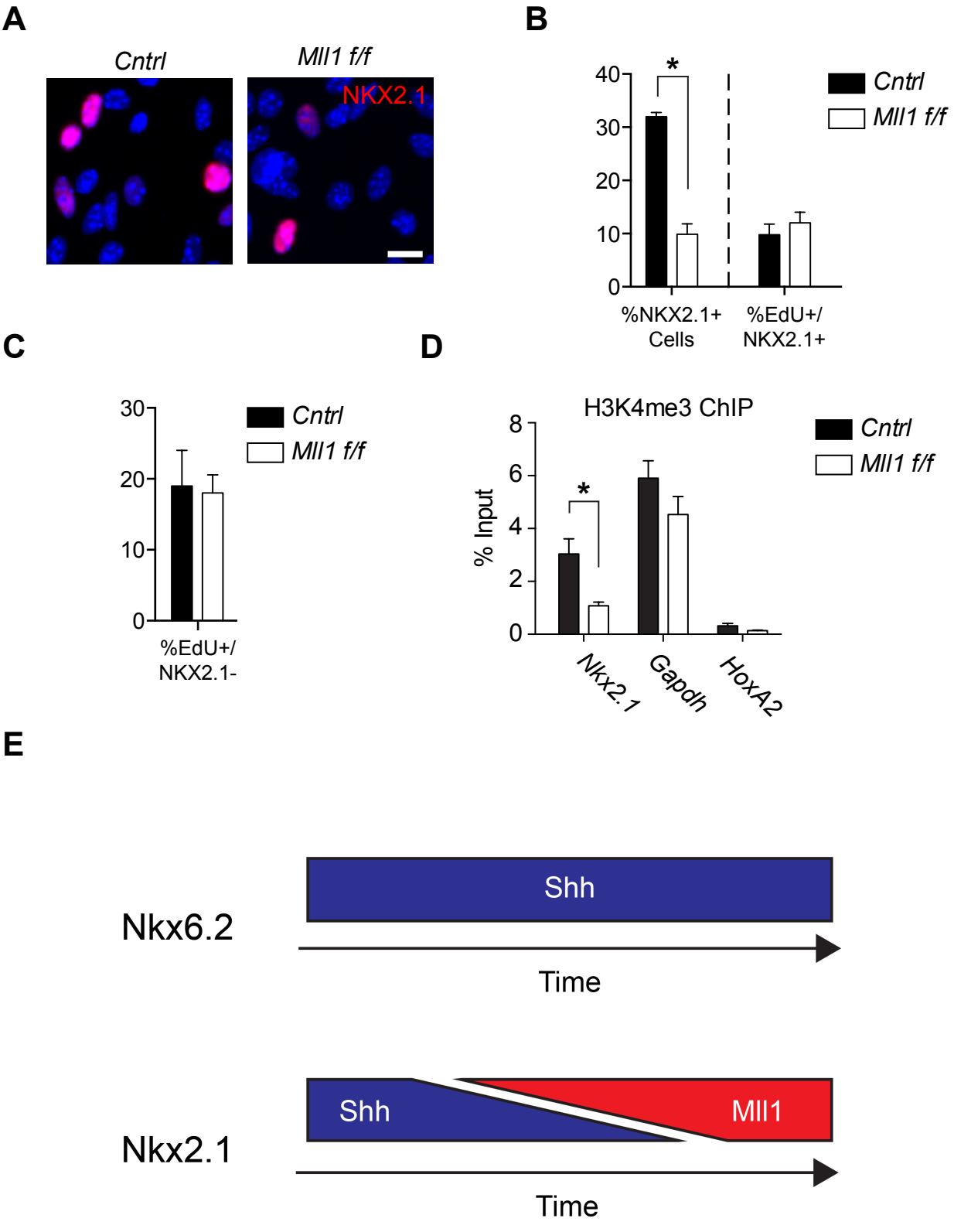
A
Cntrl
Mll1 f/f
NKX2.1
B
40
30
20
10
0
*
Cntrl
Mll1 f/f
%NKX2.1+ Cells
%EdU+/ NKX2.1+
C
30
20
10
0
Cntrl
Mll1 f/f
%EdU+/ NKX2.1-
D
H3K4me3 ChIP
% Input
8
6
4
2
0
*
Cntrl
Mll1 f/f
Nkx2.1
Gapdh
HoxA2
E
Nkx6.2
Shh
Time
Nkx2.1
Shh
Mll1
Time

Figure 3.6 The proportion of NKX2.1+ cells decrease following *Mll1* deletion. **A,** ICC analysis of NKX2.1 (red) and DAPI (blue) expression in *Cntrl* and *Mll1f/f* cultures after *Mll1* deletion. **B,** Quantification of the proportion of NKX2.1+ cells and EdU+/NKX2.1+ cells in *Cntrl* and *Mll1f/f* cultures**. C,** Quantification of EdU+/NKX2.1- cells in *Cntrl* and *Mll1f/f* cultures. **D,** q-ChIP-analysis of H3K4me3 at the Nkx2.1 promoter in *Cntrl* and *Mll1f/f* cultures. Scale bar: 10um. *=p<0.05 **E,** Diagram of *Nkx2.1* and *Nkx6.2* maintenance over time.

Figure 3.7

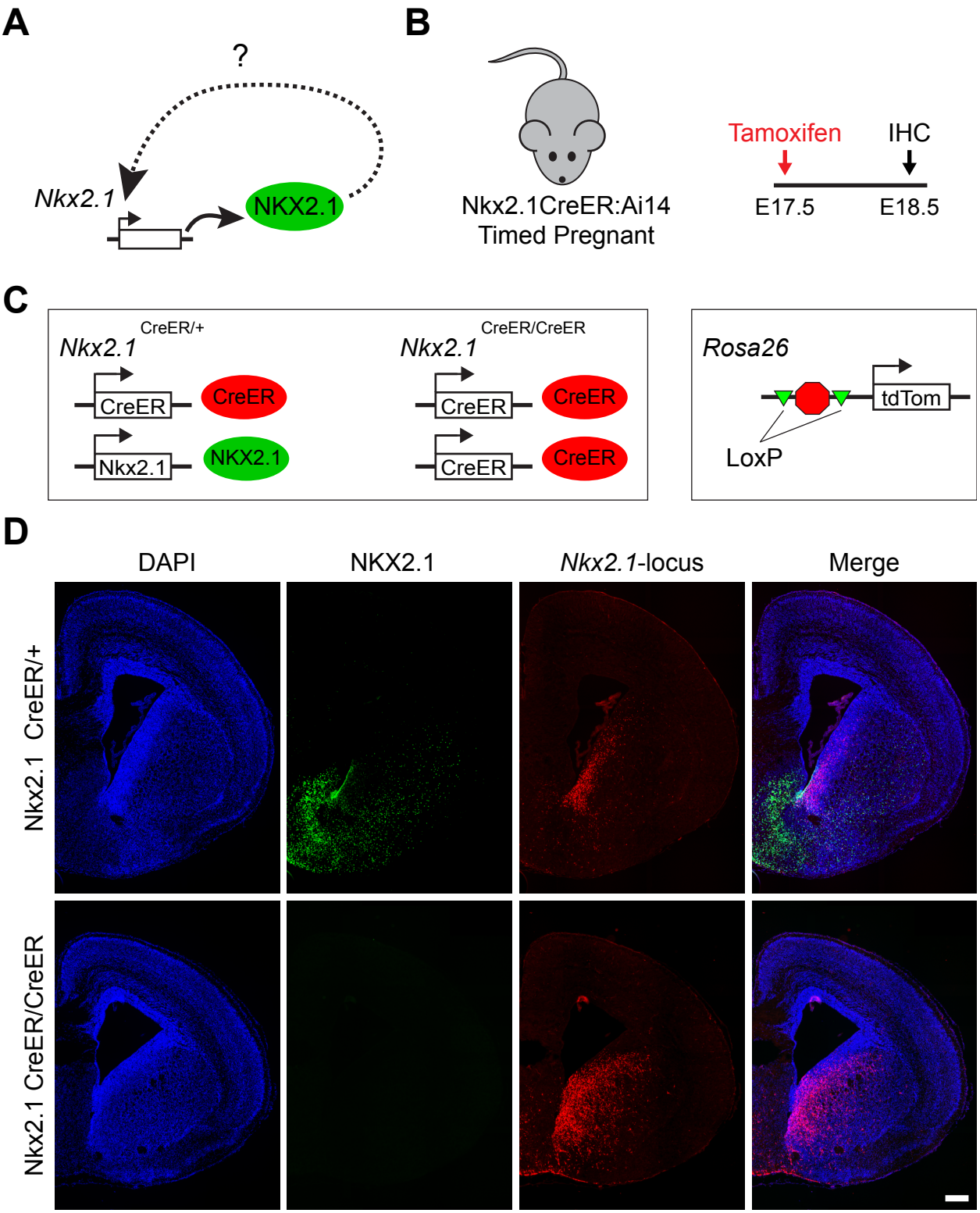
A
?
Nkx2.1
NKX2.1
B
Nkx2.1CreER:Ai14
Timed Pregnant
Tamoxifen
IHC
E17.5
E18.5
C
Nkx2.1 CreER/+
CreER
Nkx2.1
NKX2.1
Nkx2.1 CreER/CreER
CreER
CreER
CreER
CreER
Rosa26
tdTom
LoxP
D
DAPI
NKX2.1
Nkx2.1-locus
Merge
Nkx2.1 CreER/+
Nkx2.1 CreER/CreER

Figure 3.7. *Nkx2.1* expression does not require autoregulation. **A,** Schematic representation of experimental question. *Nkx2.1* locus and NKX2.1 protein (green). Dashed arrow represents a NKX2.1 protein binding the *Nkx2.1* promoter. **B,** *In vivo* labeling schematic of *Nkx2.1CreER:Ai14* timed pregnant mice. **C,** IHC analysis of NKX2.1 (green), tdTomato (red), and DAPI (blue) expression in *Nkx2.1CreER/+* and *Nkx2.1CreER/CreER* animals at similar rostral-caudal level. Scale bar: 250um.

Chapter 4: Concluding remarks and future directions

The main conclusions I present in this work are: 1) *Nkx2.1*-expressing embryonic precursors generate NKX2.1+ precursors in the adult V-SVZ 2) *Nkx2.1*+ V-SVZ precursors are neurogenic and generate a population of deep OB granule cell interneurons 3) Postnatal expression of *Nkx2.1* does not require Shh signaling 4) *Mll1* is required to maintain *Nkx2.1* expression in postnatal V-SVZ precursors. Here I discuss the larger implications and questions that these conclusions raise.

How does MLL1 maintain *Nkx2.1* expression?

Chromatin analysis indicates that MLL1 binds *Nkx2.1* regulatory elements including its promoter and putative enhancer (Figure 3.4A) suggesting that it directly regulates the *Nkx2.1* locus. Furthermore, following *Mll1* deletion, we observed a loss of H3K4me3 at the *Nkx2.1* promoter indicating that it is functioning to maintain a positive transcriptional state (Figure 3.6D). In *Drosophila,* trxG and *PcG* act antagonistically to one another (Steffen and Ringrose, 2014). Our lab has previously shown that *Mll1* is required for the expression of *Dlx2*, a master regulator of OB neurogenesis (Lim et al., 2009). In the absence of *Mll1*, repressive chromatin marks are not removed from the *Dlx2* locus, suggesting that *Mll1* is functioning to antagonize *PcG*-mediated repression of *Dlx2.* To test whether *Mll1* has a similar role in the maintenance of *Nkx2.1* expression, I could delete the PcG member EZH2 from ventral V-SVZ NSC cultures and quantify the proportion of NKX2.1+ cells. If EZH2 were antagonizing *Nkx2.1* expression, then I would anticipate that the proportion of NKX2.1+ cells would increase following EZH2 deletion. It is possible that a *PcG* member other than *EZH2* is responsible for repressing *Nkx2.1* expression. Further chromatin analysis could be performed to identify any other epigenetic modifiers that localized at the *Nkx2.1* locus.

How is MLL1 recruited to the *Nkx2.1 locus*?

In *Drosophila, PcG* and *trxG* genes are recruited to DNA elements known as Polycomb/Trithorax response elements (PREs and TREs) (Cavalli and Paro, 1998; Maeda and Karch, 2006; Simon et al., 1993). However, these sequences do not exist to the same extent in mammals (Ringrose and Paro, 2007). Furthermore, I also find that *Nkx2.1* expression does not require NKX2.1 protein (Figure 3.7), suggesting that MLL1 is not recruited to the *Nkx2.1* locus through autoregulation.

Previous studies have found that downstream mediators of the Shh require the actions of chromatin modifying enzymes to perform their functions, suggesting that there might be a direct connection between an inductive signal and the factor that maintains its transcriptional memory. For instance, Gli activator function has been shown to require the histone histone acetyltransferase PCAF in human glioblastoma cell lines (Malatesta et al., 2013). Other studies have shown that Gli repressor function requires the ATP-dependent chromatin remodeler *Brg* to prevent ectopic Shh pathway signaling in the ventral embryonic telencephalon (Zhan et al., 2011). Furthermore, Zhan et al. found that BRG occupied previously identified GLI1 binding sites (Vokes et al., 2007; Vokes et al., 2008), suggesting that Gli proteins were directly recruiting BRG to the target sites. As of now, it is difficult to assess MLL1 binding partners due to the lack of a suitably high-grade antibody. Pending the creation of such an antibody, or an endogenously expressed tagged-*Mll1* transgene, potential protein binding partners could be thoroughly assessed by mass spectrometry.

The creation of either of the aforementioned reagents would also allow for genome-wide ChIP-seq analysis, which would prove useful in identifying MLL1 binding sites and might give a clearer picture as to how MLL1 targeting occurs. Although *trxG* members do not appear to have a conserved DNA binding site in mammals, I might find that MLL1 sites are located near other known DNA-binding motifs. For instance, comparison of a MLL1 ChIP-seq dataset with known neural-specific Gli1 binding identified by McMahon and colleagues would help identify potential

overlap with the Shh pathway (Peterson et al., 2012). Futhermore, RNA-seq analysis reveals that *Mll1* deletion results in the downregulation of 799 genes including many other transcription factors involved in the development of the ventral subpallium (Figure 3.5). Knowing which of these genes were direct targets would generate a gene list that might aid efforts to identify a common targeting mechanism.

Recent studies have found that long noncoding RNAs (lncRNAs) can recruit chromatin-modifying complexes to homeotic genes. For example, in human fibroblasts the lncRNA HOTTIP is able to bind WDR5, a member of the trithorax complex, and recruit the WDR5/MLL1 complex across the HOXA locus driving gene transcription (Wang et al., 2011). HOTTIP is transcribed from the 5' end of the HOXA cluster and believed to act in *cis*. Recent lncRNA profiling strategies have identified the lncRNA *Nkx2.1-associated noncoding intergenic RNA* (*NANCI*) as a potential regulator of lung and forebrain development (Herriges et al., 2014). *NANCI* is located approximately 11 kilobases 3' of the *Nkx2.1* and is encoded on the same strand. Interestingly, Morrisey and colleagues found that *NANCI* and *Nkx2.1* expression largely overlapped in the ventral embryonic forebrain and developing lung. *In vivo* knockdown of NANCI in the developing lung significantly decreased *Nkx2.1* expression. These data suggest that NANCI could be positively maintaining *Nkx2.1* expression.

When does *Nkx2.1* expression become independent of Shh signaling?

It in unclear when in development *Nkx2.1* expression no longer requires Shh signaling. My *in vitro* culture experiments suggest that Nkx2.1 expression does not require Shh signaling postnatally. To further corroborate these findings, I analyzed brains from Gli1-nLacZ reporter mice that express nuclear LacZ from the endogenous Gli1 locus (Bai et al., 2002). High levels of canonical Shh pathway activity result in the expression of Gli1, which can be used as a "read-out" of Shh pathway signaling (Bai et al., 2004; Fuccillo et al., 2006; Garcia et al., 2010; Lee et al., 1997). At postnatal day 7 (P7), I found that LacZ expression formed a ventrodorsal gradient

across the V-SVZ, extending midway up the walls of the lateral ventricle (Figure 4.1A). While LacZ expression extended throughout the ventral tip of the lateral ventricle in the rostral V-SVZ, it was absent from the ventral-most aspect of the V-SVZ at more caudal levels where the NKX2.1+ V-SVZ domain exists (Figure 4.1B). High-resolution confocal microscopy revealed that LacZ and NKX2.1 expression were almost entirely mutually exclusive (Figure 4.1B), suggesting that high levels of Shh signaling are not present in NKX2.1+ cells. Therefore, these cells must have either lost *Gli1* expression over the course of development or derived from a population of *Nkx2.1*+ embryonic precursors that don't express *Gli1*. Further analysis of *Gli1-nLacZ* mice at earlier developmental time points would help to distinguish between these possibilities.

What are the effects of *Mll1* deletion *in vivo*?

RNA-seq analysis identified *Nkx2.1* as the second-most downregulated gene following *Mll1* deletion (Figure 3.5). *In vivo* analysis of *Mll1* deletion would provide a broader context of *Mll1*'s significance in maintaining regional transcription factor patterning in the V-SVZ. To determine if *Mll1* is required to maintain *Nkx2.1* expression *in vivo*, I would selectively delete *Mll1* from ventral V-SVZ precursors by performing a targeted injection[4] of *Cre*-expressing adenovirus into *Mll1f/f:Ai14* animals. NKX2.1 expression in the contralateral hemisphere could be used as a control to ensure that I had targeted V-SVZ precursors at the correct rostral-caudal level. I would then quantify the proportion of tdTomato+ cells that expressed NKX2.1+ over the next several months. If *Mll1* were required to maintain *Nkx2.1* expression, I would anticipate that the proportion of tdTomato+ cells that co-expressed NKX2.1 would decrease over time in *Mll1f/f* animals compared to control. López-Juárez et al. found that it took almost 2 months to observe a loss of Gsx2 expression following deletion of a conditional *Gsx2* allele and attributed this result to the perdurance of GSH2 protein and the slow rate at which V-SVZ precursors divide. If

[4] These injections are described in Merkle et al., 2007.

NKX2.1 protein has a similar half-life as GSX2 protein, I would not anticipate observing a decrease in NKX2.1+ cells for a similar amount of time (~2 months).

What are the effects of *Mll1* deletion on forebrain patterning during embryonic development? De *novo* mutations in human MLL are observed in children with Wiedemman-Steiner Syndrome (Jones et al., 2012; Strom et al., 2014). Symptoms of Wiedemman-Steiner Syndrome include short stature, developmental delay, hypertrichosis cubiti, and ocular hypertelorism. Interestingly, ocular hypertelorism is associated with increased levels of Shh signaling (Hu and Helms, 1999), a feature that we observe in *Mll1*-deleted V-SVZ NSC cultures (Figure 3.5). Taken together, these findings support the notion that *Mll1* serves to regulate Shh signaling in the brain. Moreover, mutations of other chromatin regulators are implicated in human diseases including autism spectrum disorder and schizophrenia (Ronan et al., 2013). Further investigation of the role *Mll1* and other chromatin regulators play in brain development might uncover important developmental mechanisms as well as implications for human disease.

Figure 4.1

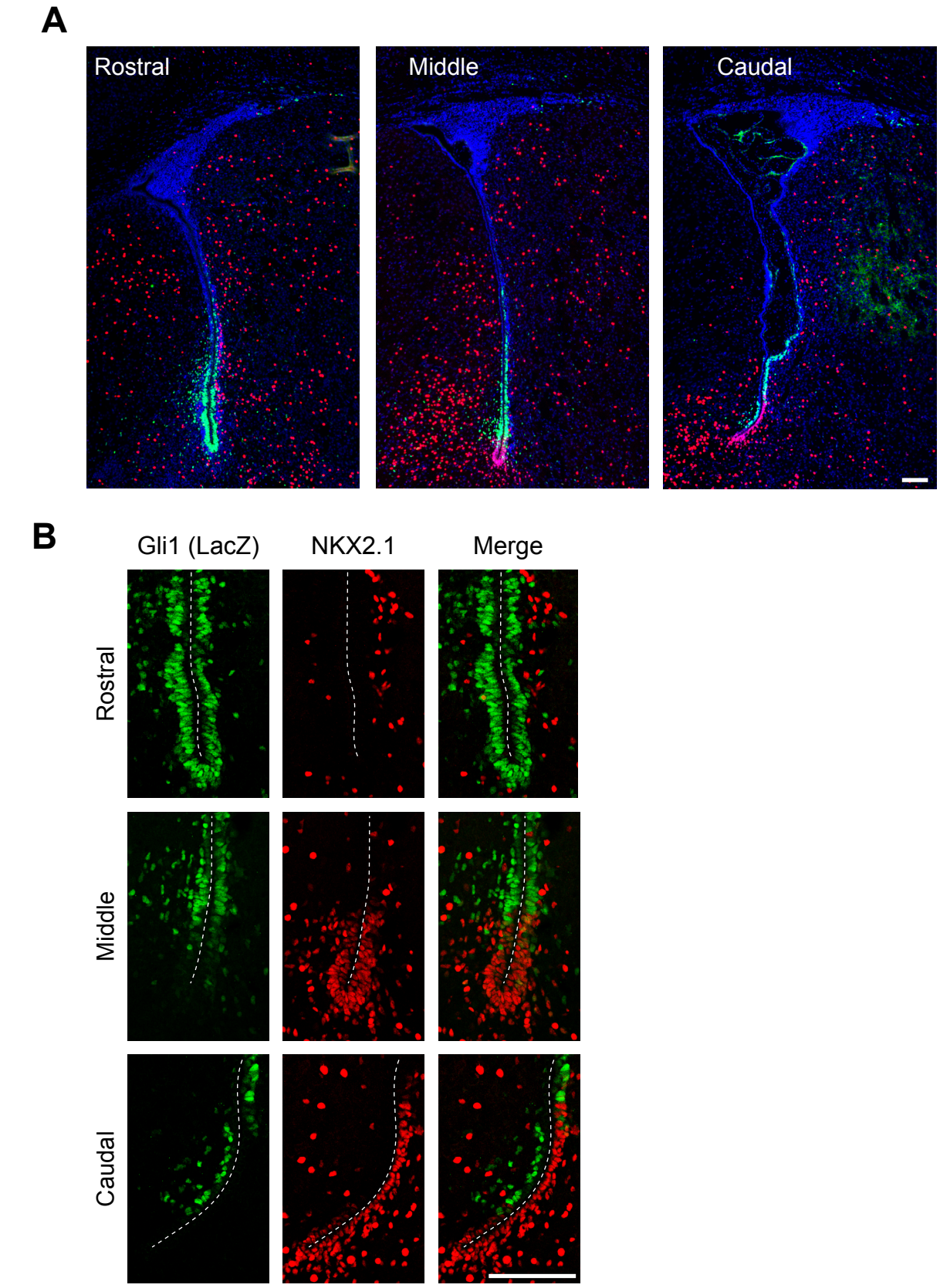
A
Rostral
Middle
Caudal
B
Gli1 (LacZ)
NKX2.1
Merge
Rostral
Middle
Caudal

Figure 4.1 The majority of NKX2.1+ cells in V-SVZ do not express Gli1. **A,** IHC analysis of β-galactosidase (green), NKX2.1 (red), and DAPI (blue) expression in P7 V-SVZ of *Gli-nLacZ* animals. β-galactosidase is expressed from the endogenous *Gli1* locus. Images are shown at Rostral, Middle, and Caudal levels of the telencephalon. **B,** High- magnification confocal images from *A.* White dashed lines depict lateral ventricle.

Scale bars: 100um.

Chapter 5: References

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