The Role of Activator E2Fs in Neural Stem Cell Activation and Exit from Quiescence

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ABSTRACT

Regenerative medicine offers tremendous potential for the treatment of irreversible damage to the brain. Activation of quiescent adult neural stem cells by clinical means to regenerate tissue can improve pathological outcomes of patients afflicted by brain trauma. Control of the cell-cycle is important in activating quiescent neural stem cells for the purpose of enhancing adult neurogenesis. Here, we uncover the role of cell-cycle regulatory transcription factors E2F1 and E2F3 in adult neural stem cell activation and characterize it. We hypothesize that the Retinoblastoma-E2F pathway is crucial for neural stem cell activation. We characterized the requirement of E2Fs1/3 for adult neural stem cells activation through a combination of multiple knockout timepoints in mice and novel markers used to identify distinct neural stem cell sub-populations. The results show a marked reduction in the neurogenic capacity of the adult brain, with common markers of proliferation and different progenitor-cell lineages decreased. Additionally, the ability of quiescent neural stem cells to transition to an active state is reduced. A whole genome-analysis of RNA isolated from E2Fs1/3-knockout adult neural stem cells has shown a shift from an active identity-state to a quiescent one. In the future, E2Fs1/3 could emerge as key regulators of quiescent stem cell activation, and thus could be potential targets for therapeutic control in order to enhance neurogenesis in patients with brain pathology.

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LIST OF ABBREVIATIONS

Ascl1: Achaete-scute homolog 1

ANOVA: Analysis of Variance

aNSC: Active Neural Stem Cell

bHLH: Brain Helix-Loop-Helix Factor

BrdU: 5-bromo-2-deoxyuridine

CD133: Prominin-1

Cdk: Cyclin-Dependent Kinase

Cre: Cyclization Recombination Protein

DAPI: 4',6-diamidino-2-phenylindole

DCX: Doublecortin

DG: Dentate Gyrus

DGC: Dentate Granule Cell

DKO: Double Knock-Out

DP: Dimerization Partner

E: Embryonic-Day

E2F: E2 Promoter Binding Factor

EGFR: Endothelin Growth Factor Receptor

EMX1: Empty Spiracles Homeobox 1

ER^{T2}: Next-Generation Estrogen Receptor Tamoxifen-2 Ligand Binding Domain

Flox: Flanked by LoxP Sites

GLAST: Glutamate Aspartate Transporter

GDNF: Glial-Derived Neurotrophic Factor

GCL: Granule Cell Layer

GFAP: Glial-Fibrillary Acidic Protein

Ki67: Ki-67 Antigen

KO: Knockout

MASH1: Mammalian Achaete Scute Homoglog 1

MEFs: Mouse Embryonic Fibroblasts

MWM: Morris Water Maze

NSC: Neural Stem Cell

OB: Olfactory Bulb

P21: Cyclin-Dependent Kinase Inhibitor 1

P27: Cycling-Dependent Kinase Inhibitor 1B

P53: Tumor Protein 53

P107: Retinoblastoma-Like Protein-1

P130: Retinoblastoma-Like Protein-2

PBS: Phospho-buffered Saline

PFA: Paraformaldehyde

PSA-NCAM: Polysialylated Neural Cell Adhesion Molecule

qNSC: Quiescent Neural Stem Cell

qPCR: Quantitative Polymerase Chain Reaction

Rb: Retinoblastoma Protein

RBPj: Recombining binding protein suppressor of hairless

RGL: Radial-Glial Like

RMS: Rostral Migratory Stream

SGZ: Subgranular Zone

Sox2: Sex Determining Region Y-box 2

SVZ: Subventricular Zone

TAP: Transit-Amplifying Progenitor

Tbr2: T-box Brain Gene 2

TKO: Triple Knock-Out

VEGF: Vascular Endothelial Growth Factor

WT: Wild-Type

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INTRODUCTION

1.1 Adult Neurogenesis

The discovery of adult neurogenesis initiated an effort to understand the mechanisms underlying generation of new neural circuitry in the brain, and its contribution to shaping behavior. Throughout one's lifetime, stem cells located in discrete neurogenic niches undergo differentiation and integration into existing neural circuits, forming connections with pre-existing processes of more mature neurons. Adult neurogenesis has been implicated in the shaping of smells, mood, memory, learning, cognition and even forgetting, throughout an organisms development (Deng et al., 2010; Frielingsdorf and Kuhn, 2007). Adult neurogenesis offers tremendous potential for regenerative medicine in the treatment of diseases such as ischaemic stroke, and neurodegenerative disorders including Alzheimer's and Parkinson's.

1.1.1 Overview of neurogenesis in the adult brain

Neurogenesis is defined as the ongoing process of generating newborn neurons during adulthood from a pre-existing pool of stem-like precursor cells. Neurogenesis is the product of precursor cell, or neural stem cell (NSC) activation by both cell-intrinsic and cell-extrinsic factors that leads to proliferation of cells that make up the neurogenic niches (Ming and Song, 2011; Toda and Gage, 2018). Precursor cells are defined here as somatic cells that are capable of replicating and terminally differentiating into a mature cell after several rounds of cell-cycle (Chagastelles and Nardi, 2011). In adult neurogenesis, a quiescent pool of stem cells located in discrete neurogenic regions of the brains undergoes intermittent activity to maintain itself and generate newborn neurons.

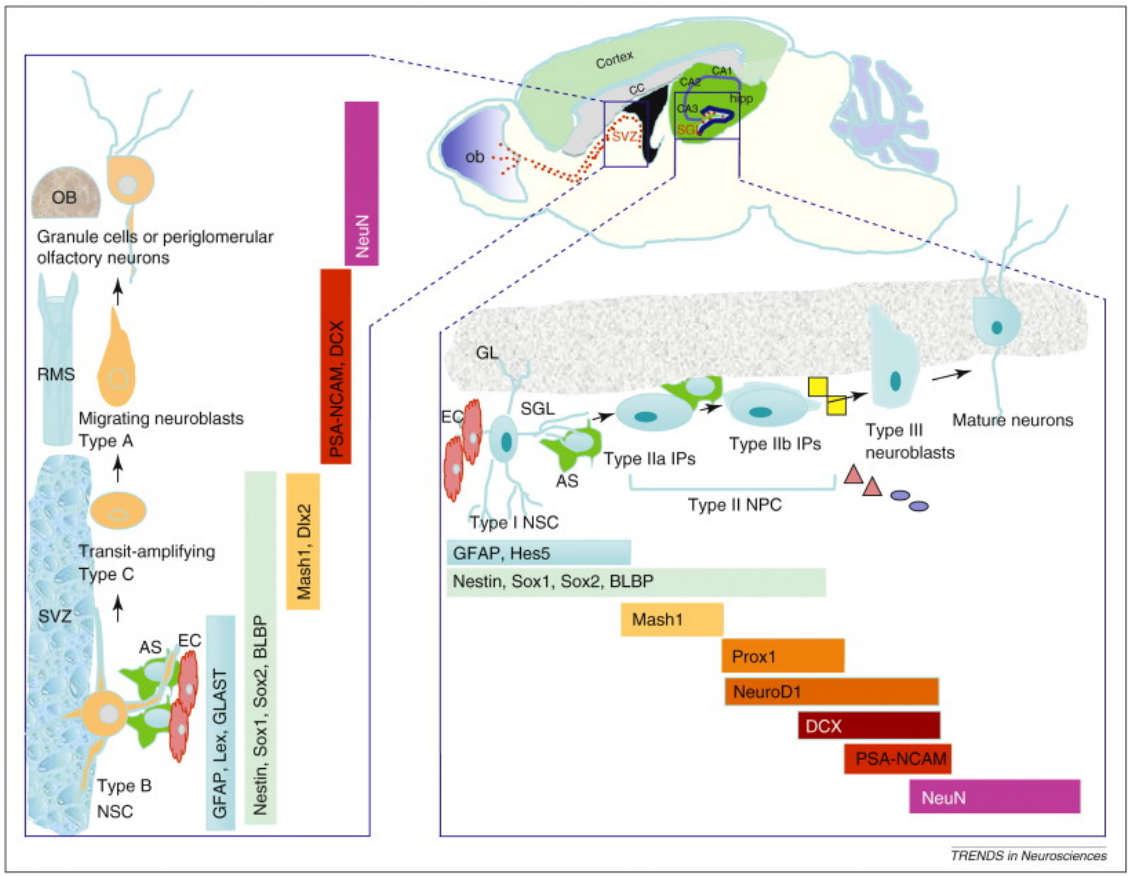
First described in rats, the process of adult neurogenesis has been well-documented in mice, songbirds, primates, and in humans (Altman and Das, 1965; Ming and Song, 2011; Reynolds and Weiss, 1992; Richards et al., 1992; Toda and Gage, 2018). The activation of stem-like precursor cells results in the generation of newborn neurons that integrate into functional circuits and can modulate memory, mood, and cognition (Akers et al., 2014; Cameron and Glover, 2015; Clemente Motta-Teixeira et al., 2015; Paton and Nottebohm, 1984). The advent of 5-bromo-2'-deoxyuridine (BrdU) has enabled researchers to mark and trace highly proliferative stem cells localized to specific niches in their journey to settle in distinct regions of the adult brain. Coupled together with techniques such as immunohistochemistry, immunofluorescence and confocal, and the discovery of stage-specific protein markers, the robustness of neurogenesis has rapidly been characterized in multitudes of organisms from birth to death. Further research revealed that neurogenesis was not a static process occurring at a constant rate, but rather a dynamic process that is heavily modulated by factors such as age, exercise, stress, environmental factors, with many such new potential intrinsic- and extrinsic-factors still being studied (Aimone et al., 2014; Ming and Song, 2011; Toda and Gage, 2018). Hippocampal adult neurogenesis has been well-characterized in most studied mammalian models, and has been proven to be a persistent, age-long process in humans (Akers et al., 2014; Boldrini et al., 2018; Spalding et al., 2013) though not without contention (Sorrells et al., 2018). Based on tracing of C^{14} incorporation as a result of nuclear bomb testing, into DNA of proliferating and newborn cells in the brain, there is clear evidence that adult neurogenesis is a lifelong process that occurs from precursor cell activation and maturation into newborn neurons (Spalding et al., 2013). However, the scale and rate of

neurogenesis may be lower than what previously was thought based on staining of newborn neurons with neural cell adhesion molecule (PSA-NCAM) marker in human brain samples (Kempermann et al., 2018; Sorrells et al., 2018).

1.1.2 The Subventricular Zone Niche

In mammals, adult NSCs are located in the subventricular zones (SVZ) and subgranular zones (SGZ) of the dentate gyrus (DG), and are multipotent cells with the capacity to become neurons, glia, or astrocytes (Figure 1) (Alvarez-Buylla and García-Verdugo, 2002; Dehay and Kennedy, 2007; Lugert et al., 2010). In the SVZ, activated precursors travel via the rostral migratory stream (RMS) into the olfactory bulb (OB) to form interneurons (Curtis et al., 2007). The integrity of both the SVZ and SGZ niches is dependent upon interaction with and signals from the vasculature and endothelial cells that line the niches (Tavazoie et al., 2008; Ward and Lamanna, 2004). For example, precursors that are cultured in the absence of endothelial factors such as vascular endothelial growth factor (VEGF) fail to proliferate and generate newborn neurons (Shen et al., 2004). These precursors possess a radial-glial like (RGL) morphology, and are typically known to express filamentous factor glial-fibrillary acidic protein (GFAP) (Figure 1). Upon activation and proliferation, these cells undergo morphological and transcriptional changes from being B-cells, to transit-amplifying progenitor (TAP) C-cells, to newborn neuron A-cells (Figure 1). The transition from each stage to the next is marked by a change in transcript makeup of a cell that allows for tracking the cell throughout its evolution. For example, T-box protein 2 (Tbr2) can mark for Type-C TAPs, whereas doublecortin (DCX) can mark for Type-A newborn neurons (Figre 1). These transitions can occur as the cells migrate rostrally from the SVZ, through the RMS,

into the OB (Figure 1). Migration occurs over the course of a couple weeks, whereby chains of cells attached through their radial-glial like morphology make their journey to the OB as neuroblasts attached to astrocytes (Lois and Alvarez-Buylla, 1994). Various guiding molecules, such as neural cell adhesion molecule (NCAM), glial derived neurotrophic factor (GDNF) and scaffolding proteins integrin and laminin assist in guiding the neuroblasts to their destination (Chazal et al., 2000; Emsley and Hagg, 2003; Paratcha et al., 2006). Upon reaching the OB, neuroblasts detach and begin locally migrating to the appropriate layer with guidance molecules such as Slit1 and receptors Robo1-3 important in radial migration as well as local axonal guidance (Nguyen-Ba-Charvet et al., 2004). Finally, neuroblasts in the OB undergo differentiation through five distinct stages to finally become interneurons with granule cell morphology (Figure 1) (Petreanu and Alvarez-Buylla, 2002). Although mostly characterized in lower order mammals as a major contributor to modulation of memory and spatial awareness, OB neurogenesis may also be a factor in human adult development (Aimone et al., 2014; Bergmann et al., 2012; Curtis et al., 2007). In rodents, generation of newborn neurons in the OB is important for discrimination between new odors, and the acquisition of new odor memory (Gheusi et al., 2000; Rochefort et al., 2002).



Lazarov et al., *Trends in Neuroscience* (2010)

Figure 1. Neurogenesis in the adult mouse brain. Precursor cell lineages and stage-specific markers allow for accurate mapping of stem cell populations.

1.1.3 The Subgranular Zone Niche

Activated SGZ precursors travel to the granule cell layer (GCL) to synaptically integrate with the CA3 region of the hippocampus (Figure 1). Evidence indicates newly integrated GCL neurons demonstrate electrophysiological properties similar, though not identical, to already established older mature neurons, suggesting neurons generated through neurogenesis are functionally similar to pre-existing neurons in those circuits (van Praag et al., 2002; Toni et al., 2007). Granular neurons generated through adult neurogenesis in the SGZ integrate into existing granule cell layers, mediating between CA3 neurons and entorhinal cortex neurons (Lugert et al., 2010). Neuronal differentiation of SGZ-derived neuroblasts is driven by GABAergic signalling that modulates maturation into neurons capable of long-term potentiation (LTP), an electrophysiological phenomena important for hippocampal memory formation (Ge et al., 2007; Tozuka et al., 2005). As in the SVZ, SGZ-derived NSCs undergo multiple differentiation stages to become neurons, with unique expression of specific proteins allowing for lineage tracing. Hippocampal neurogenesis has significant implications for spatial memory retention, navigation, pattern discrimination, forgetting, and cognition (Akers et al., 2014; Clemente Motta-Teixeira et al., 2015; Deng et al., 2010; Ming and Song, 2011). Ablation of hippocampal neurogenesis through loss of neuroblast survival is correlated with poor performance in tasks such as Morris Water Maze (MWM) after platform reversal (Akers et al., 2014; Garthe and Kempermann, 2013; Ming and Song, 2011). However, ablation of neurogenesis does not always lead to decreased performance in the MWM (Groves et al., 2013). An important function for adult neurogenesis is to establish the modulation of mood and social interaction (Lagace et al., 2010; Mak and Weiss, 2010). In experiments

of social defeat, mice with functional SGZ neurogenesis showed greater resilience to social defeat, with greater dentate granule clel (DGC) maturation and NSC activation (Becker and Wojtowicz, 2007; Lehmann et al., 2013). Depression in human subjects was shown to be linked to a lower hippocampal volume, with subjects showing poorer performance in memory recollection tasks linked with decreased hippocampal volume (MacQueen et al., 2003; Sheline et al., 1999). Other modulating factors of increased hippocampal neurogenesis include exercise and enriched environments (Fabel et al., 2009; Kempermann et al., 1997; van Praag et al., 1999, 2005).

1.1.4 Identification of NSC Lineage

Identifying the stages of stem cell development, specification, fate mapping, and differentiation is important for the purpose of studying neurogenesis and understanding how NSCs commit to certain lineages or their mode of division. Specific factors localized to adult NSCs allow for identification of stem cell lineage and tracking of different stages within NSC cell-cycle. In the SVZ, Type B cells are multipotent quiescent adult NSCs expressing GFAP, CD133, GLAST, and Sox2, and possess radial glial-like morphology with capacity for self-renewal (Figure 1) (Codega et al., 2014; Mich et al., 2014; Ming and Song, 2011). Upon activation, Type B cells enter into the cell-cycle and begin either proliferation and differentiation into rapidly-dividing Type C cells, marked by Ascl1, Tbr2, and Ki67, or decide to self-renew by creating two identical daughter cells (Figure 1) (Ming and Song, 2011; Urbán et al., 2016). Active Type C cells migrate via the rostral migratory stream (RMS) to the olfactory bulb, where they differentiate into DCX-, PSA-NCAM-positive Type A newborn neurons (Figure 1). In the SGZ, Type I cells are the quiescent, radial and horizontal glial-like NSCs, marked by Sox2 or GFAP, that undergo

selective self-renewal and fate commitment with further differentiation through either symmetric or asymmetric division (Figure 1) (Lagace et al., 2007; Lugert et al., 2010). SGZ NSCs exhibit radial glial-like morphology that is lost upon activation and transition from Type I to IIa cell type (Hodge et al., 2008, 2012; Mu et al., 2012). Type IIb cells are slower-dividing progenitors that express Tbr2 and Ascl1 are committed to a neural fate and may also begin to express Doublecortin (DCX) (Figure 1). Further differentiated cells express DCX and begin integration into the granule cell layer as Type III neuroblasts (Figure 1).

1.2 Molecular Regulation of the Niches

1.2.1 Extracellular Signaling

Similar to the SVZ NSC niche, the activation and proliferation of precursors in the SGZ niche is mediated by a wide-variety of extrinsic-factors. Recombining binding protein suppressor of hairless (RBPj) is implicated in regulation of quiescence and activation (Bjornson et al., 2012; Engler et al., 2018). As an effector of the Notch pathway, RBPj, as do downstream Notch signaling factors, hold great importance in the regulation of stem cell activity (Engler et al., 2018; Imayoshi et al., 2010; Mizutani et al., 2007). Recent studies demonstrate knockouts of Notch2 alleviate quiescence and promote cell-cycle entry, showing the importance of the Notch-pathway for maintenance of quiescence (Engler et al., 2018). Knockout studies show loss of RBPj leads to rapid depletion of the neural stem cell pool, leading to impaired neurogenic capacity in the SGZ niche (Imayoshi et al., 2010). Further studies characterizing RBPj as an effector of the Notch pathway revealed a tightly regulated pathway that is important for quiescence and activation of stem cells. Loss of Notch1 activity leads to improper stem cell

maintenance and a lack of self-renewal, as well as Notch pathway activity being unique to quiescent NSCs, but not progenitors (Aguirre et al., 2010; Alexson et al., 2006; Androutsellis-Theotokis et al., 2006; Hitoshi et al., 2002). Other important such transcription factors in NSC regulation are Ephrins and Shh signaling complexes. Lack of Ephrin or Shh activity leads to reduced cellular proliferation in the niches, and reduced survival of neuroblasts (Daynac et al., 2016; Genander and Frisén, 2010; Han et al., 2008). In contrast to regulators of NSC self-renewal and pool maintenance, a host of other factors promote differentiation into neuronal and glial lineages. The Wnt and BMP pathways have been shown to be important regulators of both neural and various glial as well as astrocytic differentiation (Bonaguidi et al., 2005; Lie et al., 2005). Other modulators of hippocampal niche activation include classical signaling molecules such as GABA, various neurotransmitters, acetylcholine, and glutamate, though the efficacy in therapeutic targeting for the purpose of tissue regeneration is unknown (Ming and Song, 2011).

1.3 Neural Stem Cell Quiescence and Activation

The majority of neural stem cells can be found in a reversible quiescent state, and can undergo activation to re-enter the cell cycle and either self-renew, commit to a neural fate, or both. Quiescence is implicated in the reduction of neurogenic capacity in the niches of adults throughout aging, and the inability of the niche to maintain itself through robust self-renewal and expansion (Encinas et al., 2011; Lugert et al., 2010; Seib et al., 2013). It is therefore pertinent for researchers in the field to find ways to not only forcefully activate NSCs from quiescence to maintain healthy neurogenesis, but to also find reliable ways to assess the state of NSCs in the niche through age. The absence of

proliferation markers such as Ki67 or PCNA in combination with cell-cycle exit markers such as p16 have been used to detect quiescent GFAP-expressing cells, though with unreliable accuracy (Urbán and Guillemot, 2014). Research to discover specific markers for quiescent (qNSC) and active (aNSC) NSCs has only relatively recently yielded a defining picture of the characteristics of niche populations (Codega et al., 2014). Novel evidence shows a sub-population of morphologically horizontal SGZ NSCs, marked by Hes Family bHLH Transcription Factor 5 (Hes5), that are comparatively primed for activation (Lugert et al., 2010; Morizur et al., 2018). This suggests there are different sub-types of adult NSCs that range in capacity to exit quiescence, with Hes5+ NSCs found in a state of activation and those lacking in Hes5 activity found in quiescence. Further characterization of this niche discovered that ubiquitylation protein HUWE1 supresses pro-neural activation of qNSCs by suppressing Ascl1 activity (Urbán et al., 2016). RNA-Sequencing shows that these qNSCs downregulate all factors related to cell-cycle, such as the Rb/E2F pathway, and upregulate factors for quiescence, such as p27 and p53 (Codega et al., 2014). Furthermore, isolated qNSCs fail to form neurospheres *in vitro*, and appear to float in culture with no visible activity, demonstrating their reduced capacity for activation when compared to aNSCs (Mich et al., 2014). Conversely, aNSCs stain positive for EGFR-related activity, and RNA-Sequencing shows higher cell-cycle activity in those cells, as well as ribosomal activity, suggesting this NSC sub-type is primed for differentiation (Codega et al., 2014; Llorens-Bobadilla et al., 2015). When cultured *in vitro*, aNSCs initiate neurosphere formation when compared to their qNSC counterparts (Mich et al., 2014). Ongoing research attempts to elucidate if there exist an even greater number of NSC sub-types, and the extent of heterogeneity among adult

NSCs. For example, under disease conditions, qNSCs can undergo further compartmentalization into qNSC1 and qNSC2, with the latter demonstrating higher Rpl1 ribosomal protein upregulation, potentially for priming towards transition into the aNSC state (Llorens-Bobadilla et al., 2015).

1.3.1 Quiescent to Active Transition Mechanisms

The switch between qNSC to aNSC transition and the pathways that underlie it are currently the topic of novel ongoing research. While many such pathways and mechanisms are currently unknown, some have been recently characterized. The Notch2 pathway and related downstream targets have been demonstrated to promote quiescence in NSCs lining the SVZ niche (Aguirre et al., 2010; Engler et al., 2018). Acute deletion of Notch2 protein driven by a Cre-Recombinase-Estrogen Receptor Tamoxifen 2 (CreERT2) driver localized to quiescent Hes5+ cells demonstrates greater PCNA+ marker activity in NSCs and TAPs that are negative for GFAP-marker, suggesting that Notch2 regulates quiescence, potentially through Hes5 downstream target (Engler et al., 2018). In those same cKO Notch2 animals, there appears to be a greater number of neuroblasts migrating to the OB, though with some depletion of the qNSC pool (Engler et al., 2018). Interestingly, acute deletion of Notch1 does not have this effect, suggesting Notch2 is crucial for qNSC maintenance. An additional target for maintenance and promotion of qNSC to aNSC transition and vice-versa is the Wnt-pathway and its non-canonical signaling roles(Chavali et al., 2018). Overxpression of Wnt5a in the SVZ demonstrates a reduction in Ki67+ proliferation among the GFAP+ population of qNSCs (Chavali et al., 2018). One target for Wnt to regulate NSC quiescence is through the Rho-GTPase Cdc42, where characterization of SVZ cultures demonstrates higher Cdc42

activity when Wnt5 is overexpressed (Chavali et al., 2018). Elucidating the mechanism controlling transition of NSCs from a quiescent to an active state and back can provide the background for development of therapies that promote acute neurogenesis while protecting the stem cell pool against depletion.

1.3.2 Ascl1 and Pro-neural Activation

Also known as MASH1, Ascl1 is a basic helix-loop-helix (bHLH) factor that has been reported to initiate differentiation in neurogenic niche NSCs with a pro-neural fate consequence. Together with Neurogenins 1 and 2 (Ngn1, Ngn2) , Ascl1 has been confirmed to be involved in pro-neural differentiation and fate specification of embryonic cells during gestation and embryogenesis (Guillemot and Joyner, 1993; Lo et al., 1991). Ascl1, localized to the ventral telencephalon during development, specifies pro-neural progenitor differentiation with a primarily GABAergic interneuron fate in the cortex during development (Casarosa et al., 1999). Sorted cortical cells from E14.5 embryos with Ngn2 and Ascl1 deleted show greater astrogenesis and reduced neurogenesis in culture after targeted differentiation assays (Nieto et al., 2001). Ascl1 is canonically downregulated by pro-quiescnece, cell-cycle exit promoting transcription factors such as the Hes-family of factors (Chen et al., 1997; Ishibashi et al., 1995). It is crucial to note that bHLH factors are the initial signaling point for differentiation into a specific fate. For example, when Ascl1 is overexpressed in carcinoma cell lines the cultured cells undergo cell cycle arrest as they transition into neurons (Farah et al., 2000). Downstream targets of this mechanism include other bHLH factors such as NeuroD1 and cell-cycle protein p27, suggesting that overexpression of pro-fate bHLH factors is sufficient to induce cell-cycle arrest as the cell matures into a neuron. Further evidence to suggest pro-neural

bHLH factors are crucial for pro-neural differentiation is described in characterization studies using in-situ hybridization where mRNA for NeuroD is found to be co-localized with BrdU pulses, suggesting that bHLH factors are persistent throughout the progenitor population into maturing neurons populations (Le Dréau et al., 2018; Lee et al., 2000; Sueda et al., 2019).

1.3.3 Pro-Quiescence versus Pro-Activation Factors

As mentioned, activity of the Hes-family of proteins has been demonstrated to suppress the cell-cycle and keep cells, as well as NSCs, in a quiescent state (Lugert et al., 2010). The study of quiescence genes and their effect on pro-neural differentiation can unlock new potential possibilities for guiding stem cell activation into a pro-neural direction. Mechanisms that regulate NSC activation via pro-neural factors can be of value in research focusing on tissue regeneration after diseases such as stroke, Parkinson's, and Alzheimer's. While Hes-factors suppress pro-neural activation of stem cells, once a cell enters the cell-cycle, controlling the balance of those factors and other pro-quiescence versus pro-neural activation factors can decide the fate of the TAP. For example, Hes5 or Hes1 overexpression in culture promotes an astrocytic fate (Ross et al., 2003). Alternatively, knockouts of pro-quiescence gene HUWE1 result in proliferating cells changes their fate from either glial, astrocytic, or neural, into an exclusively neural fate (Urbán et al., 2016). Interestingly, this process does not seem to exhaust the NSC niche of the hippocampus, suggesting that Ascl1 may be a good target for transient upregulation in tissue regeneration while maintain the health an integrity of the niche itself. Finally, in studies where mice underwent a sub-cortical stroke, the majority of NSC activation led to an astrocytic fate in the infarct area (Faiz et al., 2015). However,

retrovirus driven-Ascl1 overexpression in proliferating cells migrating to the stroke area results in a greater number of neurons settling in the penumbra region of the stroke, though their contribution to functional recovery is unknown (Faiz et al., 2015).

1.4 Birth of Adult Neural Stem Cells

The emergence of adult NSCs in the neurogenic niches is a remarkable event, as these cells possess the capacity to differentiate into multiple cell-types throughout an adults lifetime, while remaining quiescent for the majority of it. The birth of adult NSCs is reported to happen during embryogenesis, at day E14.5 of the murine gestation cycle (Fuentealba et al., 2015; Furutachi et al., 2015). Recent evidence shows that adult NSCs are born during mid-embryogenesis and undergo a transcriptional transformation from rapidly-proliferative embryonic stem cells to quiescent adult neural stem cells in both SVZ and SGZ (Fuentealba et al., 2015; Furutachi et al., 2015; Li et al., 2013). At approximately E13.5 during mouse embryogenesis, embryonic stem cells marked that are GFAP+ gradually exit the cell-cycle into a state of quiescence (G0) that persists throughout adulthood, eventually manifesting as quiescent adult NSCs. Using a novel H2B-GFP maker coupled to Histone-2B, slower-cycling cells that express Ascl1 and other pro-neurogenic markers appear in the SVZ at approximately between E13.5 and E15.5, whereas faster cycling embryonic stem cells dilute the label and continue to form the rest of the cortex (Furutachi et al., 2015). These cells persist throughout adulthood, fluxing in and out of activation within the GFAP+ population, and at times also acquire an EdU+ label together with pre-existing GFP+ label, suggesting most NSCs born during embryogenesis are able to proliferate and maintain the niche (Furutachi et al., 2015). Using a retrovirus-driven DNA library, dissected SVZ NSCs show an identical DNA-

label to fully mature neurons elsewhere in the cortex that were formed during embryogenesis (Fuentealba et al., 2015). This finding suggests that embryonic cells responsible for generating adult NSCs also generate the rest of the neurons of the cortex during embryogenesis, and that adult NSCs are born at approximately E14.5. In the ventral SVZ, these cells eventually develop radial glial-like morphology and begin to express pro-quiescence markers Neurogenin2 and HUWE1(Kele et al., 2006; Kriegstein and Alvarez-Buylla, 2009; Urbán et al., 2016). These NSCs reserve the capacity to generate newborn neurons later in the life of the adult organism upon activation.

1.5 Cell-Cycle and Rb/E2F Pathway

Regulation of cell-cycle entry and exit is a key requirement for the maintenance of a viable and healthy pool of quiescent stem cells. As mentioned, the transition from quiescence into activity within NSC subtypes may be regulated by cell-cycle proteins and related pathways. The cell-cycle can be subdivided into four continuous phases: 1) S-Phase, in which DNA is replicated and synthesized in the nucleus, 2) G1-Phase, a gap phase in which various cellular signals and responses tell the cell whether to continue with proliferation after a DNA-integrity check, differentiate, or withdraw from the cell-cycle into G0, 3) G2-Phase, in which further internal and DNA checks occur prior to division, and 4) M-Phase, known as Mitosis, in which the nucleus and cytoplasm divide (Schafer, 1998). Cyclin dependent kinases (CDKs), together with their inhibitor families INK4-family, CIP and KIP, regulate cell cycle transitions and phases once a cell has exited G0 (Leemans et al., 2011; Vanderluit et al., 2007). Cdk2-null mice display a lack of proliferation and progenitor cell loss as neurogenesis declines with age, demonstrating decreased BrdU+ and Ki67+ staining in the SVZ niche and

along the RMS (Jablonska et al., 2007). Cdk2 regulates neurogenesis in the niche by upregulating Cdk4, necessary for NSC self-renewal in the SVZ niche (Jablonska et al., 2007). Indeed, Cdk2-overexpression in germline knockout Cdk4 mice leads to a rescue of proliferation in the SVZ (Jablonska et al., 2007).

Unlike stem cells, mature neurons are terminally differentiated and cannot re-enter the cell-cycle successfully, and are high in expression for cell-cycle proteins promoting G0-phase and permanent withdrawal from the cell-cycle (Andrusiak et al., 2012; Aranda-Anzaldo, 2012; Frade and Ovejero-Benito, 2015). Other important regulators of the cell-cycle include The Cip/Kip-family of CDK-inhibitors. Members of this family, such as p27 and p21, appear to be crucial effectors of cell-cycle entry by inhibition of CDK2/Cyclin-E (Soos et al., 1996). The role of the INK4-family of cell-cycle effectors, particularly p16 (INK4a), is crucial in proper regulation of cell-cycle by inhibiting CDKs/Cyclins that induce cell-cycle progression (Sherr and McCormick, 2002). For example, p16-deficient mice appear to develop tumors at a higher frequency when compared to their wild-type counterparts, and tumorigenesis is exacerbated in p16-mutants after exposure to carcinogenic compounds (Krimpenfort et al., 2001; Serrano et al., 1996). Alternative reading-frame transcription of the p16 genetic locus leads to generation of another tumor suppressor, p19, which appears to selectively activate p53 (Kamijo et al., 1997). Activation of p53 generally occurs in response to sources of mass DNA-mutation, DNA-damage, and tumorigenic activity, leading to upregulation of genetic targets that result in apoptosis or cell-cycle arrest (Levine, 1997). The canonical cell-cycle pathway may therefore play an important role in the regulation of NSC activation.

1.5.1 Role of Cell-Cycle in Neurogenesis

The length of cell-cycle phases impacts the mode of division for stem cells, and can affect whether the cell undergoes self-renewal or differentiation. Overexpression of growth factors and endothelial signaling molecules during embryogenesis shortens the length of G1-phase to promote re-entry into the cell-cycle and promote proliferation over differentiation (Hodge et al., 2004; Lukaszewicz et al., 2002). Conversely, prolonging G1-phase can accelerate differentiation and aberrantly change the fate of activated stem cells (Calegari and Huttner, 2003; Salomoni and Calegari, 2010; Vernon et al., 2003). Changing the length of G1-phase can also impact migration of neuroblasts, suggesting that cell-cycle length is an incredibly delicate mechanism that has remarkable impact on stem cell activation and commitment (Nguyen et al., 2006). Cdk2/4 DKO mice display an increased G1-phase leading to differentiation over proliferation, suggesting these specific CDKs are needed for NSC self-renewal (Jablonska et al., 2007). Furthermore, Cdk6 mutant mice show aberrant stem cell proliferation in the DG and SVZ, with thinner lateral walls lining the VZ and a thinner SGZ. This effect is conserved through regulation of G1-phase length (Beukelaers et al., 2011). In contrast to canonical Cdk-regulation of cell-cycle length affecting self-renewal or differentiation decisions, Cdk5 is implicated in regulation of proper neuroblast migration through the SVZ and maturation of neuroblasts (Hirota et al., 2007; Jessberger et al., 2008). Retrovirus-driven knockdown of Cdk5 in migrating neuroblasts leads to reduced spine formation during dendritic arborization and prevents mature neurons from properly integrating in GCL of the hippocampus (Jessberger et al., 2008). In addition, conditional knockout of Cdk5 using an EMX1-Cre driver leads to inability of TAPs and neuroblasts to traverse the RMS, and therefore a

lack of DCX+ newborn neurons in the OB following knockout (Hirota et al., 2007). Cell fate-mapping and lineage is largely decided prior to TAP migration, during activation, and decided by a variety of cell intrinsic- and extrinsic-factors (Dehay and Kennedy, 2007). Activation of the majority of CDKs is dependent upon co-binding with D-type cyclins, cyclins D1, D2, and D3, with the exception of CDK5. Germline deletion of Cyclin-D2 leads to a malformed hippocampal formation and a thinner SGZ formation, with BrdU+-proliferation reduced in the niche throughout the animal's lifetime (Kowalczyk et al., 2004). Overall, the classical cell-cycle, its phases and cues have important roles in NSC activation, proliferation, and lineage decisions.

1.5.2 Retinoblastoma (Rb) Gene-Family Overview

One important regulator and effector of the cell-cycle is the Rb/E2F-Pathway, potentially regulating hundreds of important downstream cell-cycle dependent targets (Fischer and Müller, 2017). Initially identified and first cloned in pediatric retinal tumor disease research, retinoblastoma (Rb) has been demonstrated to effect G1-phase arrest(Friend et al., 1986; Lee et al., 1987; Weinberg, 1995). Deletion of Rb has been implicated in the formation of aberrant tumors through excessive ectopic cellular proliferation, with reintroduction of Rb into cancerous tissue shown to slow down and effectively stop tumor growth (Harbour and Dean, 2000; Huang et al., 1988). Rb possesses two functional "pocket" domains A and B, with a spacer region between them, which interact together to bind targets on the genome, together with the E2F-family of transcription factors (Sage, 2012). In humans, most oncogenic retinal activity due to Rb-dysfunction occurs after mutations are found to disrupt the pocket-binding domain and prevent Rb from binding transcriptional targets (Harbour, 1998; Horowitz et al., 1990).

Rb activity in the cell is regulated by its phosphorylation and dephosphorylation. Rb protein possess 16 potential sites for phosphorylation by various CDKs and Cyclins to potentiate its regulation and repression of the cell-cycle (Harbour and Dean, 2000). Rb prevents transition from G1/S-phase, or prevents entrance into the cell cycle from G0/G1. Such crucial activity is demonstrated in studies where deletion of Rb induces re-entry of the cell-cycle both *in vivo* and *in vitro* (Chen et al., 2004; MacPherson et al., 2004). Progressive phosphorylation of Rb by CDKs and Cyclins, such as Cdk4, Cdk6, Cyclins D1-D3, and Cyclin E, can relieve Rb repression of downstream E2F-targets to enact G1 to S-phase transition promoting cell-cycle re-entry (Choi and Anders, 2014; Narasimha et al., 2014). Phosphorylation of Rb is not a single event, but a continuous concert of multiple cell-cycle effectors working to keep the cell-cycle active. Studies have shown that Cdk4/6 phosphorylate Rb to enact early G1-phase continuation into G1/S-Phase checkpoint where Cdk2/Cyclin-E phosphorylates Rb into S-phase where Cdk2 overtakes regulation of Rb (Harbour et al., 1999; Sherr, 1996; Sherr and Roberts, 1999). Numerous Rb-binding proteins possess an LXCXE domain, such as common HDACs, allowing them to co-activate targets with Rb. One such potential target for Rb is repression of the genes responsible for Cyclin E transcription, where overexpression of Cyclin E in tissues that contain a phospho-resistant form of Rb is able to facilitate cell-cycle re-entry by overcoming Rb's repressive activity (Leng et al., 1997; Lukas et al., 1997). Disruption of the Rb-HDAC complex by Cdk4/6/Cyclin-D allows for expression of Cyclin-E which can induce cell-cycle re-entry to G1-phase and continuation of the cell-cycle (Zhang et al., 2000). Rb performs many of its repressive actions on the cell-cycle together with

another family of proteins, the E2F-family of transcription factors (Iaquinta and Lees, 2007; Sage, 2012).

Similar homologous pocket-proteins p107 and p130 also act as oncorepressors and bind E2Fs to promote cell-cycle repression. As the case with Rb, p107/p130 also act as oncosupressor factors, inhibiting E2Fs and their activity as well as suppressing the cell-cycle when overexpressed in tissue (Claudio et al., 1994; Starostik et al., 1996; Zamanian and La Thangue, 1993). Pocket-proteins have been shown to selectively bind different members of the E2F-family of transcription factors (Sardet et al., 1997). One potential role for p107 and p130 is to produce potential tissue-specific redundancy for cell-cycle regulation against Rb-mutations (Dannenberg et al., 2004). It is important to note that the activity of Rb on cell-cycle repression and re-entry is tissue-specific. For example, conditional deletion of Rb in pancreatic B-cells does not seem to produce cell-cycle activation despite levels of p107 and p130 remaining the same compared to healthy B-cells (Vasavada et al., 2007). However, in human retinoblastoma disease or murine pituitary gland cancer a single deletion of Rb is sufficient to induce ectopic proliferation (Dannenberg et al., 2004). When heterozygote Rb-mice are crossed with either p107 or p130 knockouts, tumors appear to develop in a host of tissues in the body, such as lungs, ovaries, thyroid, and bone (Dannenberg et al., 2004). Mutations of p130 in small-cell lung cancer cell line GLC2 or in glioblastoma cell-line T98G demonstrate its role in oncogenesis and may lend to the idea that while pocket-proteins are similar in function, they are no entirely redundant (Claudio et al., 1994; Helin et al., 1997). E2F-complexes with either Rb, p107, or p130 are differentially constructed depending on which stage of the cell-cycle proliferating cells are in. For example, Rb/E2F complexes are found

preferentially in G1/S-Phase, whereas p130/E2F or p107/E2F complexes are quiescent, differentiated, or S-phase cells (Dyson, 1998). This stage-specific complex formation is further compartmentalized by the specific E2F-family factor bound to the relevant pocket-protein. For example, binding of p130 to E2F4 is important for and abundant in quiescent cells, whereas Rb binds E2F1-4 throughout G1/S-Phase transitions (Corbeil et al., 1995; Lacy and Whyte, 1997; Qin et al., 1995). Therefore, it appears the pocket-proteins possess overlapping oncosuppressor functions in a tissue-specific manner, though with some crucial specificity. While Rb, p107, p130 share similarity in both function and pocket-binding activity, some key differences impact the role each protein has in the cell. An important role for regulation of the cell cycle at the G1 to S-phase transition DNA-integrity checkpoint by pocket proteins, p107 and p130 (Dannenberg et al., 2004; Hurford et al., 1997; McClellan et al., 2007; Vanderluit et al., 2007). Studies show that a triple-knockout (TKO) of Rb-family proteins Rb, p107, and p130 results in ectopic proliferation and differentiation of the adult NSC pool through deregulation of E2Fs (Julian et al., 2013; Vanderluit et al., 2007). As such, The Rb/E2F pathway is important for cell-cycle regulation and the promotion of cell-cycle entry by NSCs.

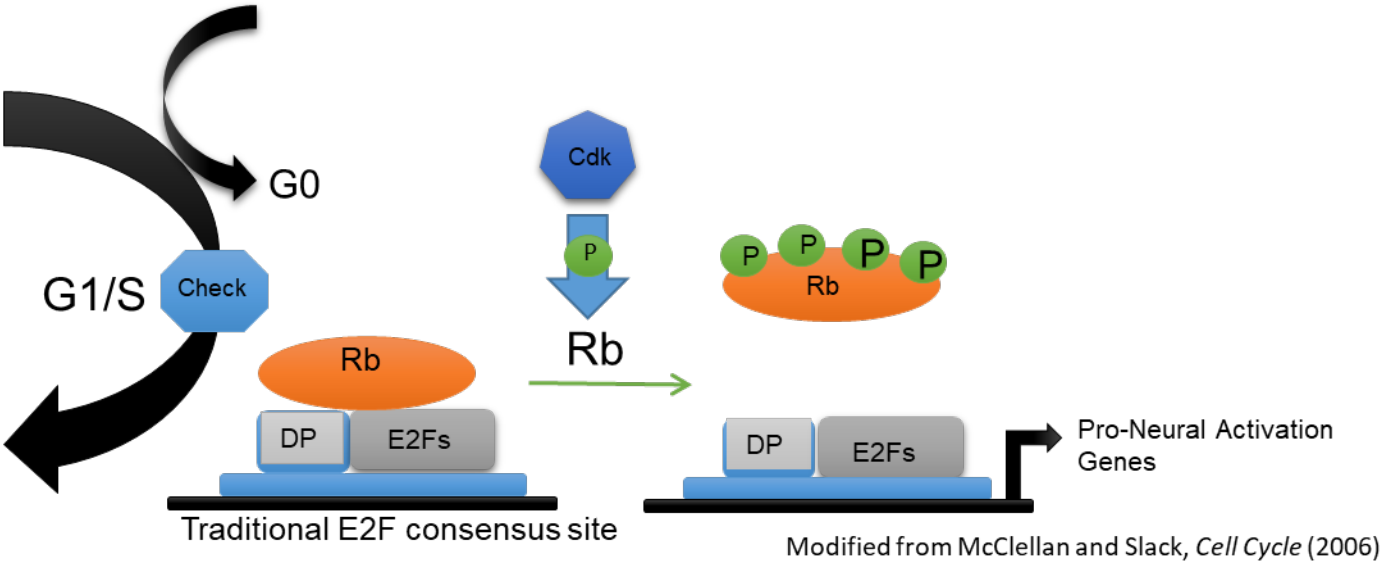


Figure 2. Canonical Rb-E2F pathway within the context of stem cell activation.

1.5.3 E2F Transcription Factors Overview

Initially discovered after studies looking at tumor-initiating DNA-viruses identified proteins associated with excessive proliferation, the E2Fs have been found to be highly conserved in evolution, with 8-family members appearing in mammalian biology(Chen et al., 2009b). Generally, there are 8 distinct E2F-proteins, some grouped based on similar function: 1) E2Fs1-3 are considered activator E2Fs and function to enact cell-cycle progression by expressing great binding affinity to targets that promote cell-cycle activity once relieved from Rb repression, 2) Repressor E2Fs4-6 which act to repress the transcription of cell-cycle proteins at the G0/G1-Phase transition out of quiescence (E2Fs4-5) or at the S-Phase checkpoint (E2F6), and 3) Atypical E2Fs7-8 able to bind DNA without DP protein and whose function may be to promote stem cell differentiation or senescence (Aksoy et al., 2012; Chen et al., 2009b; Iaquinta and Lees, 2007; McClellan and Slack, 2007; Ramirez-Parra et al., 2004). It is important to note there are two isoforms of E2F3 – E2F3a and E2F3b which may have distinct and opposite roles in certain cell-types (Julian et al., 2013). The E2F-family of transcription factors are an important transcriptional target for the Rb-family proteins (Figure 2). Physical interaction of E2Fs with all three pocket-proteins Rb, p107, and p130 is required for regulation of G1/S-Phase transitions and DNA replication (Dyson, 1998; Nevins, 2001; Sherr and McCormick, 2002). Canonical Rb/E2F complex has been shown to regulate the cell-cycle at the G1/S-Phase checkpoint though this mechanism is tissue- and context-specific (Qin et al., 1995; Zhu et al., 1993). For example, presence repressor E2Fs4/5 and pocket-protein complexes bind to transcriptional targets to keep a proliferating cell at quiescent G0-stage, while E2Fs1-3 complexes with pocket-proteins

are characterized in proliferating and differentiating cells (Ikeda et al., 1996; Moberg et al., 1996). E2Fs heterodimerize with dimerization partner (DP) to effect G1/S phase transition and cell-cycle progression once de-repressed by phosphorylated Rb (Figure 2). Experiments where a dominant negative isoform of DP1 is inserted into cultured human osteosarcoma cells demonstrate an arrest of proliferation, suggesting DP is required for binding of the Rb/E2F complex to its targets (Wu et al., 1996). Transition from a quiescent G0-state to G1- and onwards involved upregulation of activator E2Fs1-3 activity and downregulation of repressor E2Fs (Iaquinta and Lees, 2007; Trimarchi and Lees, 2002). To support this, overexpression of E2F1 in Rat-2 fibroblasts on in primate kidney cells is a sufficient signal to promote S-phase entry, albeit with higher apoptotic activity (Kowalik et al., 1995; Qin et al., 1994). It is important to note that the canonical Rb-E2F binding interaction does not involve an LXCXE binding activity, but rather the pocket and a carboxy-terminal region affinity binding activity (Huang et al., 1988; Lee et al., 1987). However, studies implicating E2F1 as a tumor-suppressor rather than an oncogene demonstrate tumor formation in mouse reproductive tracts, lungs, and among T-cell lymphocytes, suggesting that E2F1, and potentially other E2Fs, can have opposite roles in different tissues (Field et al., 1996; Yamasaki et al., 1996). The E2F-Family of transcription factors possesses a key role in the induction and repression of the cell-cycle and may therefore be a potentially important player in quiescence and activation of cells.

In addition to cell-cycle entry and proliferation, E2F activity can also regulate apoptotic mechanisms. Indeed, while many tumors display high levels of runaway-E2F activity, they are also marked with significant apoptosis and senescence (Field et al., 1996; Martinez et al., 2010; Wu et al., 2009). Virus-driven overexpression of E2F1 in

REF-52 fibroblast cells results in higher S-phase-bound DNA synthesis that correlates with increased apoptosis (Kowalik et al., 1995). Interestingly, this effect can buffered when fibroblasts obtained from p53-mutant mice are infected with the same construct, suggesting that E2F1 activates apoptosis through the p53 pathway (Hsieh et al., 1997; Kowalik et al., 1995). However, p53 is not always necessary for the apoptotic function of E2F1, which can act through Apaf1 or SIRT1 to also induce apoptosis (Moroni et al., 2001; Phillips et al., 1997; Wang et al., 2006). Human WI-38 cells infected with E2F1-overexpressing retroviruses showed a flattened morphology reminiscent of senescent cells, in addition to having higher Senescence-Activated Beta-Galactosidase (SABG) staining when compared to normal or E2F1-null cells (Dimri et al., 2000). Overphosphorylation of Rb leading to high free E2F1 activity has been implicated in higher apoptotic activity in cells marked by increased TUNEL and Active-Caspase 3 (AC3) activity in mouse pituitary tissue (Kowalik et al., 1995; Lazzerini Denchi and Helin, 2005). Following inactivation of p53, the tumor growth accelerates with a large reduction in apoptotic activity, suggesting the Rb/E2F pathway is crucial for healthy regulation of the cell-cycle through interaction with p53 and other CDKs. Other activator E2Fs may also play a role in activation of apoptotic pathways. Irradiated E2F3-mutant mouse embryos showed higher TUNEL staining in the developing CNS when compared to E2F3-heterozygotes, in addition to cultured cells showing a similar result, suggesting E2F3 is required for apoptosis in developing cells (Martinez et al., 2010). Overall, activator E2Fs have secondary functions as pro-apoptotic genes in response to excess levels of DNA-synthesis, though this function is highly tissue-specific and cell-specific.

1.5.4 Role of E2Fs in Stem Cells – Overview

Further characterization of E2F activity in various tissues and cellular populations reveals that E2Fs may have a key role in progenitor cell activation, proliferation, and differentiation (Chong et al., 2009; Fajas et al., 2002; Müller et al., 2001; Sage, 2012; Sangwan et al., 2012). Depending on the tissue, E2Fs1-3 may regulate proliferation and survival of progenitor cells (Chong et al., 2009; Wu et al., 2001a). In Rb-deficient tissues that are tumor prone, additionally knocking out E2F1 is sufficient to stall tumor growth, suggesting that E2F1 regulates proliferation of progenitor cells under ectopic growth (Sangwan et al., 2012). However, triple knockouts of the activator family of E2Fs1-3 has no effect on the proliferation of embryonic stem cells (ES cells), retinal progenitors, and on gut stem cells suggesting the requirement of the activator E2Fs is both stage- and stem cell niche-specific (Bertoli et al., 2013; Chong et al., 2009). In mice with conditional Rb knockout and germline p107 knockout, hematopoetic stem cells show loss of quiescence, aberrant activation, and an overwhelming differentiation towards a myeloid cell fate while producing overwhelming E2F3 activity (Viatour et al., 2008). Therefore, E2Fs may not only be required for healthy proliferation of stem cells, but also critical cell-fate decisions (Julian and Blais, 2015). Interestingly, E2Fs1-3 can promote exit from quiescence and subsequently switch to having repressive activities to promote elongation of G1-Phase and potentiating the cell to differentiation cues for longer, deciding its fate (Chen et al., 2009a; Chong et al., 2009; Lange and Calegari, 2010). In the brain, NSC activation and choice between proliferation or differentiation may be regulated by E2F3 depending on which isoforms is expressed, through regulation of stemness marker Sox2 (Julian et al., 2013). Overall, while Rb/E2F regulation of stem cell proliferation,

differentiation, and cell-fate decisions is a relatively new and emerging field, there seems to be a case to be made for the importance of E2F-activity for the regulation of stem cell quiescence.

1.5.5 Role of E2Fs in the Central Nervous System - Overview

Emerging research into role of E2Fs in the CNS has begun to identify several interesting mechanisms in the regulation of neurogenesis. Activator E2F activity may have essential requirement for the proliferation and differentiation of adult NSCs and progenitors (Calegari et al., 2005; Julian et al., 2013; McClellan and Slack, 2007; Wu et al., 2001b). Overall numbers of E2F3 mutant mouse embryonic fibroblasts (MEFs) that were allowed to grow in culture appear recover slower after treatment with H3-Thymidine (Humbert et al., 2000). The same mutant E2F3 MEFs also appear to cycle and proliferate less. E2F1 knockout show similar phenotypes, with reduced NSC proliferation in the SVZ niche, and increased apoptosis in migrating neuroblast populations, though little to no effect in peripheral nervous system neurons, suggesting that E2F1 activity is not only tissue-specific, but both context- and cell-subtype-specific (Field et al., 1996; Tsai et al., 1998; Wu et al., 2001b). Dysregulation of Cdk/Cyclin activity that affects Rb/E2F can either lengthen or shorten G1-Phase leading to improper balance between progenitor population numbers and newborn neuron survival in the CNS (Lange et al., 2009; Lim and Kaldis, 2012). Chromatin immunoprecipitation (ChIP-on-chip) experiments demonstrate that activator E2F3a directly binds to transcriptional sites of pro-quiescence gene Hes5 as well as pro-neural gene e.g. Ascl1, suggesting there may be E2F3 regulation of these targets (Julian et al., 2013). Loss of either E2F1 or E2F3 can lead to a reduction in differentiative or proliferative capacity of NSCs or their migration

(Höglinger et al., 2007; Julian et al., 2013; McClellan et al., 2007; Zyskind et al., 2015). Proper regulation of activator E2Fs1/3 is crucial for prevention of ectopic proliferation. Tumor-prone Rb knockout tissue can be rescued by additionally deleting activator E2F3, suggesting that controlling activator E2Fs activity is perhaps essential for suppression of potential oncogenes (Rotgers et al., 2014; Symonds et al., 1994). The Rb/E2F pathway also has additional roles in apoptosis. In experiments where Rb is deleted, mice experience tumor growth along the lateral SVZ, albeit with a high rate of apoptosis (Symonds et al., 1994). Taken together, E2F-family transcription factors are crucial targets for the regulation of the cell-cycle and for G0-G1-S-Phase transition. Research into stem cell activation dynamics together with E2F-family transcription factors presents an exciting path for contributing to the field of neural tissue regeneration.

RATIONALE, HYPOTHESIS & AIM

The arguments presented demonstrate a case for the need to study activation and transition of qNSCs to aNSCs. It is possible to conclude that pro-neural differentiation factor Ascl1 has the capacity to not only potentially wake dormant NSCs and force them into the cell-cycle, it may also divert the fate of those cells to a neural one (Faiz et al., 2015; Kim et al., 2011; Urbán et al., 2016). As stated, pro-quiescence and cell-cycle proteins could be the major suppressant and indeed a barrier to Ascl1 activity. Therefore, establishing a link between cell-cycle proteins, such as the E2F-family of transcription factors, to pro-neural activation factors like Ascl1 could potentially provide meaningful conclusions about the regulation of quiescent NSC transitions into a pro-neural active state. The mechanisms that regulate qNSC to aNSC transitions are yet to be fully characterized, and the presence of Ascl1 seems to promote pro-neural activation in a variety of NSC sub-types. This mechanism is yet to be fully elucidated, and with novel sub-types of NSCs being discovered in different contexts (e.g. stroke-specific sub-types), it is imperative to elucidate the key regulators involved. The specific requirements for E2Fs in this transition is yet to be investigated. Based on preliminary data from our lab, significant binding of E2F3 on the promoter region of Ascl1 via ChIP-on-chip analysis shows E2Fs may have a regulatory site for pro-neural activation factors (Julian, Blais & Slack, *Unpublished*). In addition to this, previous characterization of mice lacking E2F1, E2F3, or both in the neurogenic niches shows a significant reduction in adult neurogenesis with lower Ki67+ progenitor and DCX+ newborn neuron populations as seen in Figure 1 (Ahmadi et al., *Unpublished*; Vandenbosch et al., *Unpublished*). We therefore present a rationale wherein E2Fs1/3 have a unique and regulatory role in the

Ascl1-driven activation of NSCs. I therefore hypothesize that **activator E2Fs1/3 have a requirement for neural stem cell exit from quiescence and entry into the cell-cycle.** To test this, I propose the following primary aim:

Primary Aim: To characterize the requirement of E2Fs1/3 for neurogenesis in both neurogenic niches of the brain.

MATERIALS AND METHODS

2.1 Mice

Several transgenic lines of mice were used in this study: E2F1$^{-/-}$ transgenic mice were crossed onto E2F3$^{F/F}$ mutant mice on a Nestin-Cre^{ERT2} background. The mouse colony was maintained on a mixed background (C57BL6/S129/FVBN) (Charles River Laboratories, Wilmington, MA). E2F1-null mice are a kind gift from Dr. Michael E. Greenberg through Dr. David S. Park (Field et al., 1996). E2F3-floxed mice are a kind gift from Dr. Gustavo Leone. Briefly, 32 kb loxP restriction sites were inserted into mouse ES-cells to span exon 3 (Wu et al., 2001a). When targeted by Cre-recombinase, the exon 3 fragment is deleted resulting in a non-functional E2F3 protein unable to bind DNA and enact cell-cycle progression. The Nestin-CreERT2 mice were obtained from Dr. Suzanne J. Baker (St. Jude Children's Research Hospital) (Cicero et al., 2009). Breeding of animals used for this study is as follows: For characterization analysis at any timepoint, males homozygous for E2F1-mutant alleles, homozygous for E2F3-mutant alleles, and possessing Nestin-Cre^{ERT2} (E2F1$^{-/-}$ E2F3$^{F/F}$ Nestin-Cre^{ERT2+}) were crossed to females heterozygous for E2F1-mutant alleles, homozygous for E2F3-mutant alleles, and lacking Nestin-Cre^{ERT2} (E2F1$^{+/-}$ E2F3$^{F/F}$ Nestin-Cre^{ERT2-}) to produce control progeny (E2F1$^{+/-}$ E2F3$^{F/F}$ Nestin-Cre^{ERT2-}) and potential DKO-progeny (E2F1$^{-/-}$ E2F3$^{F/F}$ Nestin-Cre^{ERT2+}). For RNA-Sequencing, mice were crossed such that either controls (E2F1$^{+/-}$ E2F3$^{F/+}$ Rosa26-YFP$^{F/+}$ Nestin-Cre^{ERT2+}) or DKO (E2F1$^{-/-}$ E2F3$^{F/F}$ Rosa26-YFP$^{F/+}$ Nestin-Cre^{ERT2+}) Nestin-CreERT2 for the purpose of driving conditional Rosa26-YFP expression.

All experiments were approved by the University of Ottawa Animal Care Committee (ACVS). Mice were genotyped using Sigma Extract-N-Amp kit (Sigma) and primer designs for Control-Cre, E2F1, E2F3 according to manufacturer's protocol (Table 1).

2.2 Tamoxifen Administration

Briefly, Estrogen-activated Cre-recombinase is localized to Nestin-expressing cells throughout the organism. Upon administration of Tamoxifen (TAM) and binding of TAM to estrogen-receptors on the Cre-recombinase protein results in translocation of the Cre to the nucleus and begins its actions to seek out and cut loxP restriction sites. In our study, adult mice 6-12 weeks of age were administered daily 100ul TAM by oral gavage at a concentration of 50mg/mL for 5-consecutive days (Khacho et al., 2016).

Gene	Genotyping Primers
E2F1	E2F1-5 5'-GGATATGATTCTTGGACTTCTTGG-3' E2F1-3 5'-CTAAATCTGACCACCAAACGC -3' PGKB 5'-CAAGTGCCAGCGGGGCTGCTA AAG-3'.
E2F3	Primer A 5'-GTGGCTGGAAGGGTGCCAAG-3' Primer B 5'- TGAATCATGGACA GAGCCAGG-3' Primer C 5'-GATTGATTCTGGGTTGTCAGG-3'
Nestin-CreERT2	Cre 3 5'–TTGCCCCTGTTTCACTATCCAG-3' Cre 5 5'–TGCTGTTTCACTGGTTATGCGG-3'
	qPCR Primers
E2F1	E2F1-F CTGCAGCAACTGCAGGAGAG E2F1-R CTCCGAAAGCAGTTGCAGCTG
E2F3	E2F3-F AAACGCGGTATGATACGTCCC E2F3-R CCATCAGGAGACTGGCTCAG
Ascl1	Ascl1-F GCAACCGGGTCAAGTTGGT Ascl1-R GTCGTTGGAGTAGTTGGGGG

Table 1. Genotyping and qPCR primer sequences used

2.3 Perfusion, Fixation, and Cryosectioning

Mice were euthanized with 30ul of 1mg/kg IP injection of Euthanyl (Sodium Pentobarbital – supplied by ACVS uOttawa) followed with cervical dislocation. Mice were then perfused with 0.9% cold saline by inserting a perfusion needle into the left ventricle and making an incision in the right atrium. Following this, mice were perfused with cold, fresh 4% paraformaldehyde (PFA) (pH 7.4), and then brains were carefully dissected and bathed in vials containing approximately 20 mL of 4% PFA overnight. 24 hours following this, brains were washed in 1% PBS and transferred into 20% sucrose, 0.3% sodium azide in 1% PBS cryoprotectant solution for 48 hours. After this, brains were frozen at approximately -35^{o}C in isopentane solution cooled with dry-ice chunks and instantly placed into a temperature-proof container filled with dry-ice nuggets. For cryosectioning frozen brains were encased in Tissue-Tek OCT Compound (Sakura) inside a cold (-22^{o}C) cryostat and then cut at 30 um serial sections after being mounted in a coronal orientation. Cut tissue was stored in wells containing 0.01% sodium azide in 1% PBS solution indefinitely and covered with parafilm to prevent evaporation of solution.

2.4 Immunofluorescence

In general, cut tissue was washed 3x in 1% PBS solution for 5-minutes each wash, then placed in 0.1% Triton-X-100, 0.1% Tween-20 in 1% PBS solution with diluted primary antibody at indicated concentrations (Table 2) for 24-hours in 4C. The next day, the tissue was washed 3x in 1% PBS solution for 5-minutes each wash, and then placed in 0.1% Triton-X-100, 0.1% Tween-20 in 1% PBS solution with diluted secondary antibody at indicated concentrations (Table 2) for 2-3 hours in room temperature.

Following this, tissue was washed one more time for 10-minutes in 1% PBS solution before being mounted on slides and cover-slipped with Immunomount (Genetex).

2.5 Imaging and Cell Quantification

Imaging was done on a Zeiss LSM800 confocal microscope at 20x with tiling and Z-stacking. Every section on a slide, for both SVZ and SGZ, was imaged and quantified with 3 Z-Stacks followed with a SUM projection in Fiji (Schindelin et al., 2012) post-processing. For counting, manual cell-counting was done via the cell-counter module in Fiji. In brief, every cell lining the SVZ or SGZ was counted after a line was drawn outlining the lateral ventricles and stem cell niche of the internal SGZ no more than 5um away from the niche (Kim et al., 2011; Urbán et al., 2016). Every required permutation and combination of cells was counted independently of each other by combining or subtracting the right channels. Total cell counts per SVZ or SGZ per section were summed together for all sections, and multiplied by the number of total wells (9 wells) into which the brain was cut, to provide an estimate of the total number of cells per structure. A 1-way ANOVA was performed to obtain a significance value with an alpha of 0.05 with HET and DKO mouse' relevant regions as independent variables and various cellular permutations as dependent variables.

Antibody	Animal	Dilution	Purpose	Source
Ki67	rabbit	1:500	IF	Cell Marque, SP6
Tbr2	rat	1:250	IF	eBioscience, 14-4875-82
Sox2	goat	1:500	IF	Santa Cruz
Sox2	goat	1:500	IF	Neuromics, GT15098
Sox2	mouse	1:250	IF	R&D Systems, MAB2018
Sox2	rabbit	1:250	IF	Millipore, AB5603
DCX	GP	1:5000	IF	EMD Millipore, AB2253
MASH1	mouse	1:200	IF	BD Biosciences, 556604
GFAP	mouse	1:5000	IF	Chemicon Intl, MAB3402
EGFR	rabbit	1:100	IF	Abcam, ab52894
CD133	rat	1:50	IF	eBioscience, 14-1331-82
Nestin	goat	1:5000	IF	Novus Biologicals, NB100-1604
EAAT1	rabbit	1:500	IF	Abcam, ab416
Aldolase-C	rabbit	1:250	IF	Novus Bio, NBP1-90954
SSEA-1	mouse	1:5	FACS	BD Bioscience, 560120
GFP	chicken	1:1000	IF	Abcam, ab13970
In-Vivo-EDU	-	-	IF	BaseClick, BCK647-IV-IM-L

Table 2. Primary antibodies used for immunofluorescence

2.6 Flow Cytometry

Whole brains from adults were obtained from animals and subsequently microdissected for the SVZ. Briefly, the tissue was then manually sliced with a sharp scalpel blade, followed with Papain digestion (Cedarlane Labs). After a wash, the suspension was taken to the flow-cytometer (Moflo Astrios, Beckman Coulter) and sorted for YFP-signal. The resulting supernatant was spun at 3000 rpm for 3 minutes to pellet the cells, and frozen in -80 following aspiration for RNA-extraction. For embryonic dissections, a similar approach was utilized. Briefly, the ventral VZ was micro-dissected with a set of scalpel blades, and manually triturated with a pipette tip. Following this, the suspension was digested with trypsin (Sigma-Aldrich) and quenched with trypsin-inhibitor (Roche). After a wash, the single-cell suspension was stained with 647-conjugated SSEA-1 (CD15) antibody (BD Bioscience, 1:5). The stained suspension was subsequently taken to the flow-cytometer and sorted for 647-positive signal. The resulting supernatant was spun at 3000 rpm for 3 minutes to pellet the cells, and frozen in -80 following aspiration for RNA-extraction. All RNA was extracted using the PicoPure Kit (Thermo-Fisher) according to manufacturer specifications and protocol.

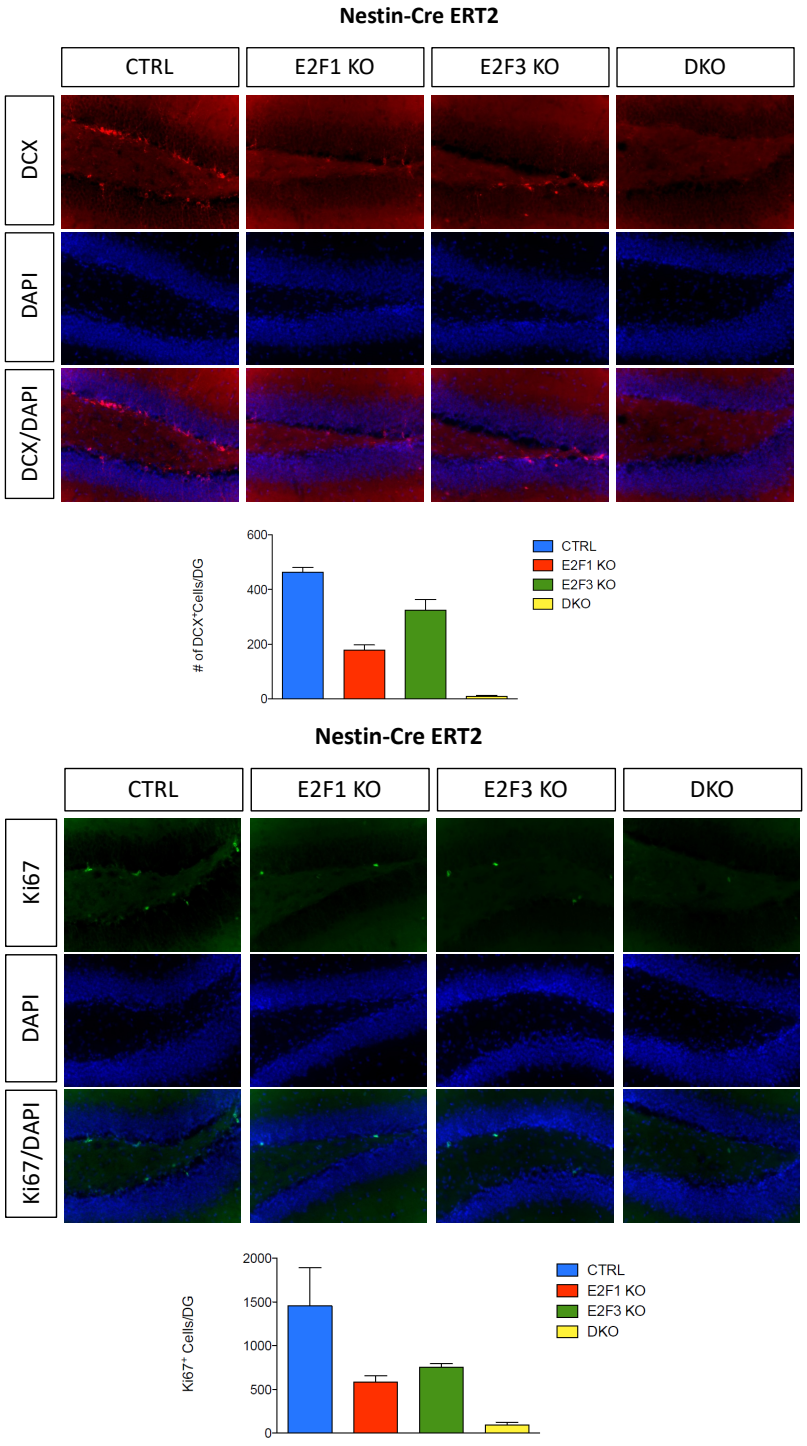
2.7 Quantitate Polymerase Chain Reaction (qPCR) and RNA-Sequencing

Equal amounts of RNA were quantified by nanodrop (1 ug/uL) were loaded into samples ready for qPCR. The SyberGreen RT-PCR Kit (Qiagen) was used to mix the samples and then the samples were processed in the RotorGene Q Amplifier (Qiagen). Resultant expression values were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and standardized to control HET mice values. Program using Rotor Q gene qTPCR was as follows: hold for 10 mins at 55^{o}C for reverse transcription, then hold for 5 mins at 95^{o}C for initial PCR activation step, cycling for 40 cycles for 5 sec at 95^{o}C, which is the denaturation step, melt ramp 50 to 99^{o}C, rising 1 degree each step for combined annealing/extension, wait for 90 sec pre-melt, then wait for 5 sec afterwards.

RESULTS

3.1 Characterizing the requirement of E2Fs1/3 in NSC niche neurogenesis

We set out to characterize the requirement of activator E2Fs1/3 for adult neurogenesis. Our foray into the importance of E2Fs1/3 for adult neurogenesis began after we characterized the requirement for Retinoblastoma (Rb) and Rb-family proteins in the neurogenic niches of the brain (Vandenbosch et al., 2016). Previous characterization results from our lab show that lack of germline E2F1 and acute deletion of E2F3 at adulthood using a Nestin-Cre driver results in a marked reduction in markers of newborn neurons (DCX+) and proliferation (Ki67+) in the adult brain (Figure 3) (Ahmadi et al., *Unpublished*). Additionally, ChIP-Seq experiments from our lab revealed significant binding between the activator isoform of E2F3 (E2F3a) and Sex-determining region Y-box 2 (Sox2), a marker used to denote stem cell population number in our characterization studies (Julian et al., 2013). With this characterization data at our disposal, we sought to explore the relationship between activator E2Fs1/3 and the regulation of neurogenesis. More specifically, we asked the question whether activator E2Fs1/3 are required for generation of newborn neurons in the adult brain.



(Ahmadi, Vandenbosch & Slack, *Unpublished*)

Figure 3. **Double-knockout of E2F1 and E2F3 in embryogenesis of dorsal telencephalon disrupts formation of the SGZ and reduces neurogenesis**
(A) Quantification of total Sox2+ cells, TAPs marked by Tbr2, newborns neurons marked by DCX, and proliferation marked by Ki67 of 16-week old adult mice either heterozygous (HET) for E2F1 and E2F3, sKO for E2F1 or E2F3, and DKO for both E2F1 and E2F3 driven by NestinCre-ERT2 recombinase. Significant reduction in total DCX+, and Ki67+ cells between either HET, E2F1KO, E2F3KO and DKO (***p<0.001, ***p<0.001 respectively) in a 1-way ANOVA. Significant reduction in total DCX+ population between HET and E2F3KO (*p<0.05) in a 1-way ANOVA. **(B)** Representative images taken using LSM 510 microscope with 20x objective from 30 um free-floating coronal sections of 10-week old animals stained for Sox2, Tbr2, DCX, Ki67, and DAPI (n=3).

We targeted E2Fs1/3 for deletion due to prior studies demonstrating the ability of E2F1 and E2F3 to partially compensate for each other with both appearing to have similar roles and functions in the regulation of adult NSCs, and deletion of either resulting in a moderate decline on neurogenesis (Chong et al., 2009; Cooper-Kuhn et al., 2002; Julian et al., 2013). Following this pattern, we surmised that if both E2F1 and E2F3 were to be mutated then NSC activation and neurogenesis could be severely impaired. Being that E2Fs are crucial for embryonic brain development, we reasoned that germline deletion of E2F1 while keeping E2F3 intact will allow for proper embryogenesis to occur due to activator-E2F overlapping roles, and only an acute deletion of E2F3 in NSCs in adulthood would be sufficient for impairment of neurogenesis (Attwooll et al., 2004; DeGregori and Johnson, 2006; King et al., 2008; Parisi et al., 2007; Wu et al., 2001a). Using a NestinCre-ERT2 driver, we performed several knockout timepoints that represent the time after administration of Tamoxifen resulting in floxed-E2F3 excision, effectively leading to DKO Nestin+ cells. We asked if E2Fs1/3 are required for neurogenesis in the neurogenic niches of the adult brain at 2, 4, and 8 weeks after tamoxifen administration when compared to control. To answer our question of the impact of E2Fs1/3 deletion on NSC activation and adult neurogenesis in general, select markers for NSC sub-types and differentiated cells were used for immunofluorescence. We began by staining for Doublecortin (DCX) a marker for newborn neurons with a distinct morphology depicting arborization and a cytoplasmic presence. Additionally, we decided to co-label every progenitor stain with the proliferation marker Ki67 to assess the proliferative capacity of the progenitor sub-type population we targeted. We found that after staining both the Subventricular Zone (SVZ) and Subgranular Zone (SGZ) for DCX

as well as co-labeling with proliferation marker antigen-Ki67 (Ki67), a gradual reduction and both total and proliferative numbers of DCX was observed throughout the timepoints. DCX was significantly down 2, 4, and 8 weeks ($p<0.01$, $p<0.01$, $p<0.001$ respectively) (Figure 4B) after tamoxifen administration as well as Ki67/DCX co-labelling ($p<0.01$, $p<0.001$, $p<0.001$ respectively), with an 85% reduction in total DCX population between HET control and 8-week DKO in the SVZ (Figure 4B'). We asked if a similar phenotype could be observed in the SGZ niche. Total DCX population lining the SGZ showed a similar pattern, with a significant reduction at 2, 4, and 8-weeks ($p<0.05$, $p<0.001$, $p<0.001$ respectively) (Figure 5B) after Tamoxifen administration as well as marked decrease in Ki67/DCX co-labelling ($p<0.001$, $p<0.001$, $p<0.001$ respectively) (Figure 5B').

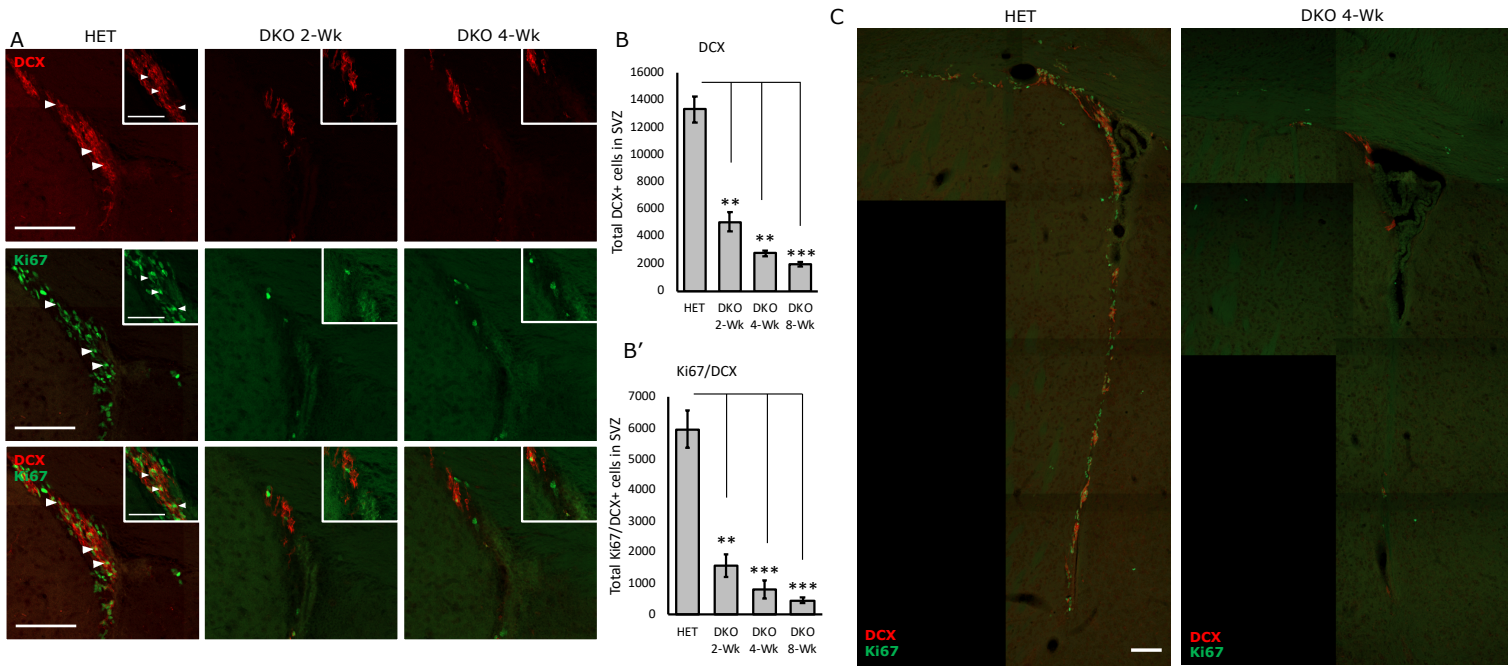


Figure 4. **E2fs1/3 Deletion Impairs Neurogenesis In The Subventricular Zone Of The Adult Brain.**
(A) Representative confocal images of total newborn neurons (DCX+) and their proliferation (Ki67+/DCX+) in the adult SVZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Arrowheads show DCX+ and DCX+/Ki67+ co-localized cells.
(B) Quantification of total newborns neurons (DCX+) in the adult SVZ at either baseline levels (HET), 2-weeks (DKO 2-Wk) or 4-weeks (DKO 4-Wk) after E2Fs1/3 DKO. Significant reduction in DCX+ cells between HET and DKO 2-Wk (**p<0.01), HET and DKO 4-Wk (**p<0.01), HET and DKO 8-Wk (***p<0.001) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing. **(B')** Quantification of total newborns neurons (Ki67+/DCX+) in the adult SVZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk), or 8-weeks (DKO-8Wk) after E2Fs1/3 DKO. Significant reduction in Ki67+/DCX+ cells between HET and DKO 2-Wk (**p<0.01), HET and DKO 4-Wk (***p<0.001), HET and DKO 8-Wk (***p<0.001) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing.
(C) Representative confocal images of total newborn neuron proliferation (Ki67+/DCX+) in the entire adult SVZ at either baseline levels (HET) or 4-weeks (DKO 4-Wk) after E2Fs1/3 DKO.

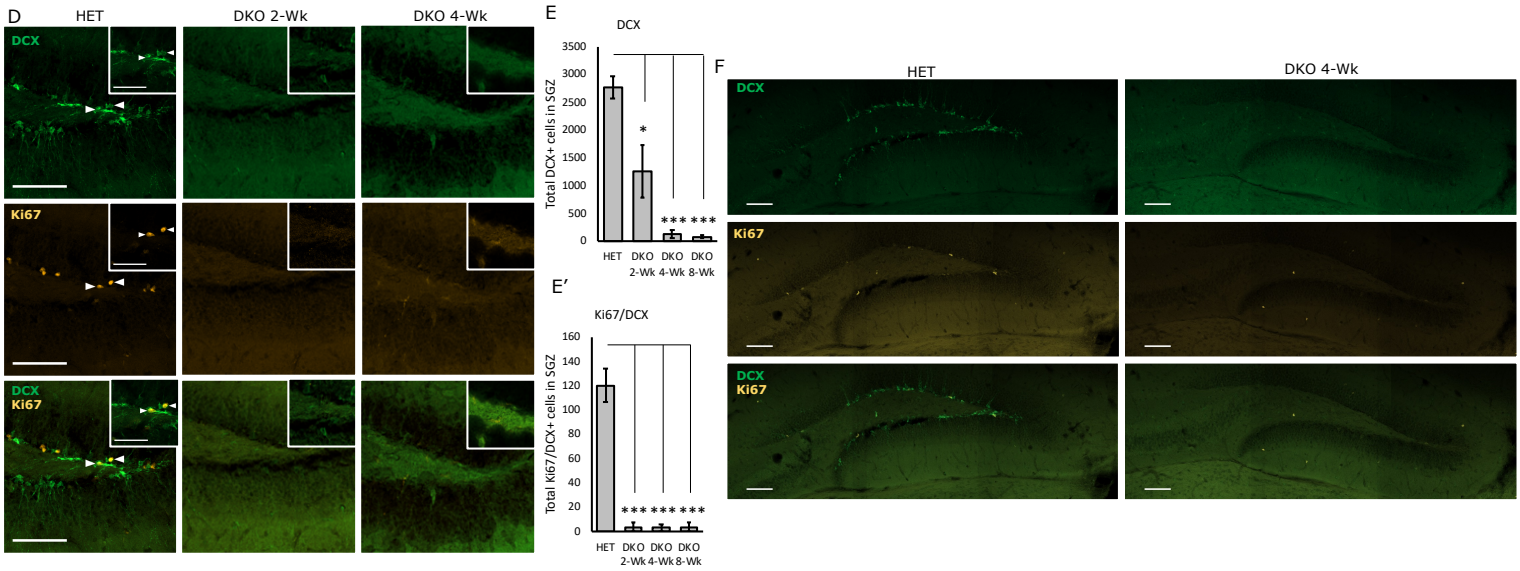


Figure 5. **E2fs1/3 Deletion Reduces the Newborn Neurons In The Subgranular Zone Of The Adult Brain.**
(A) Representative confocal images of total newborn neurons (DCX+) and their proliferation (Ki67+/DCX+) in the adult SGZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Arrowheads show Sox2+ and Sox2+/Ki67+ co-localized cells.
(B) Quantification of total newborn neurons (Sox2+) in the adult SGZ at either baseline levels (HET), 2-weeks (DKO 2-Wk) or 4-weeks (DKO 4-Wk) after E2Fs1/3 DKO. Significant reduction in DCX+ cells between HET and DKO 2-Wk (*p<0.05), HET and DKO 4-Wk (***p<0.001), HET and DKO 8-Wk (***p<0.001) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing. **(B')** Quantification of total newborn neurons (Ki67+/DCX+) in the adult SGZ at either baseline levels (HET), 2-weeks (DKO 2-Wk) or 4-weeks (DKO 4-Wk) after E2Fs1/3 DKO. Significant reduction in Ki67+/DCX+ cells between HET and DKO 2-Wk (***p<0.001), HET and DKO 4-Wk (***p<0.001), HET and DKO 8-Wk (***p<0.001) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing.
(C) Representative confocal images of total newborn neurons (DCX+) and their proliferation (Ki67+/DCX+) in the entire adult SGZ at either baseline levels (HET) or 4-weeks (DKO 4-Wk) after E2Fs1/3 DKO.

3.2 Characterizing the role of E2Fs 1 and 3 in progenitor proliferation and differentiation

We then asked if there may be a defect in differentiation and commitment, prompting us to look at earlier neural lineages and look at transit-amplifying progenitor (TAP) populations marked by T-box brain protein-2 (Tbr2), Achaete-scute Family BHLH Transcription Factor-1 (Ascl1) and more primitive cellular populations marked with Sox2 (Brazel et al., 2005; Hodge et al., 2008; Kim et al., 2011; Zhang and Jiao, 2015). Sox2 here is used to mark for and count undifferentiated progenitors. We used Tbr2 and Ascl1 to mark for progenitors no longer in a quiescent state, but in a more advanced, differentiated state that is highly active and proliferative as the progenitor matures to become either neuron, astrocyte, or glia. In the SVZ, Ascl1was significantly reduced 2, 4, and 8-weeks ($p<0.05$, $p<0.01$, $p<0.01$ respectively) (Figure 6B) after Tamoxifen administration in DKO mice compared to HET mice, with a 90% decrease in total Ascl1 TAP population by 8-weeks. Additionally, proliferation of Ascl1 progenitors marked with Ki67/Ascl1 co-labeling was also significantly reduced ($p<0.01$, $p<0.01$, $p<0.01$) (Figure 6B'), suggesting that both TAP population and its proliferative capacity is decreased following compound E2Fs1/3 DKO in Nestin cells, with a 94% loss in Ki67/Ascl1 co-labeling by 8-weeks post DKO. Similar observations were made in the SGZ, where Tbr2 was used to mark TAPs and track their proliferation. In the SGZ, Tbr2 was significantly reduced 2, 4, and 8-weeks ($p<0.01$, $p<0.01$, $p<0.01$ respectively) (Figure 7B) after Tamoxifen administration in DKO mice compared to HET mice, with a 96% decrease in total Tbr2 TAP population by 8-weeks. Additionally, proliferation of Ascl1 progenitors marked with Ki67/Tbr2 co-labeling was also significantly reduced

($p<0.01$, $p<0.001$, $p<0.001$) (Figure 7B'), suggesting that both TAP population and its proliferative capacity is decreased following compound E2Fs1/3 DKO in Nestin+ cells, with a 95% loss in Ki67/Tbr2 co-labeling by 8-weeks post DKO. We concluded that there is a significant and rapid loss of highly-proliferative progenitor populations in both neurogenic niches of the adult brain, and their proliferative capacity is decreased.

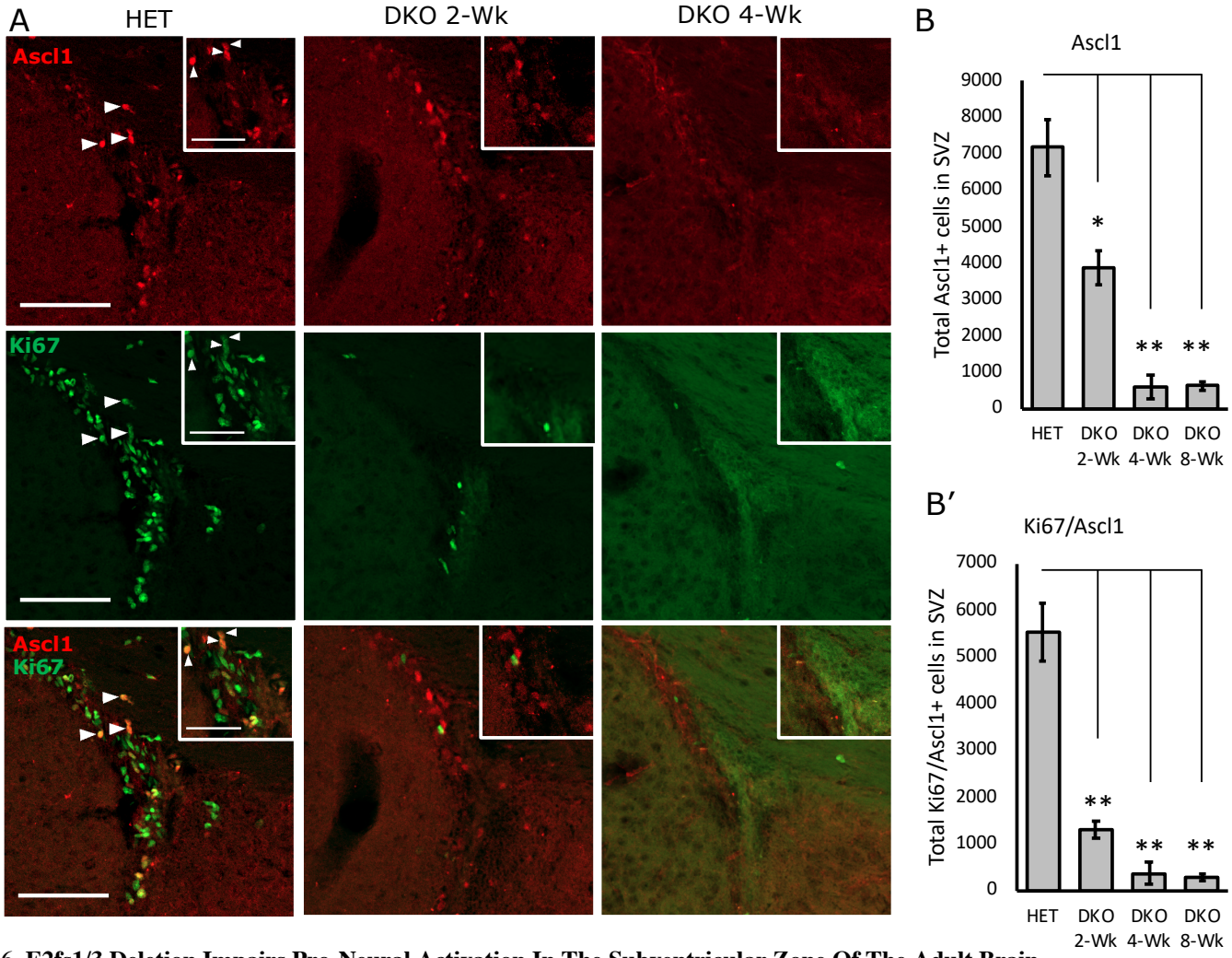


Figure 6. **E2fs1/3 Deletion Impairs Pro-Neural Activation In The Subventricular Zone Of The Adult Brain.**
(A) Representative confocal images of total pro-neural active NSCs (Ascl1+) and their proliferation (Ki67+/Ascl1+) in the adult SVZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Arrowheads show Ascl1+ and Ascl1+/Ki67+ co-localized cells.
(B) Quantification of total pro-neural active NSCs (Ascl1+) in the adult SVZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk) or 8-weeks (DKO8-Wk) after E2Fs1/3 DKO. Significant reduction in Ascl1+ cells between HET and DKO 2-Wk (*$p<0.05$), HET and DKO 4-Wk (**$p<0.01$), HET and DKO 8-Wk (**$p<0.01$) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing. **(B')** Quantification of total pro-neural proliferating NSCs (Ki67+/Ascl1+) in the adult SVZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Significant reduction in Ki67+/Ascl1+ cells between HET and DKO 2-Wk (**$p<0.01$), HET and DKO 4-Wk (**$p<0.01$), HET and DKO 8-Wk (**$p<0.01$) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing.

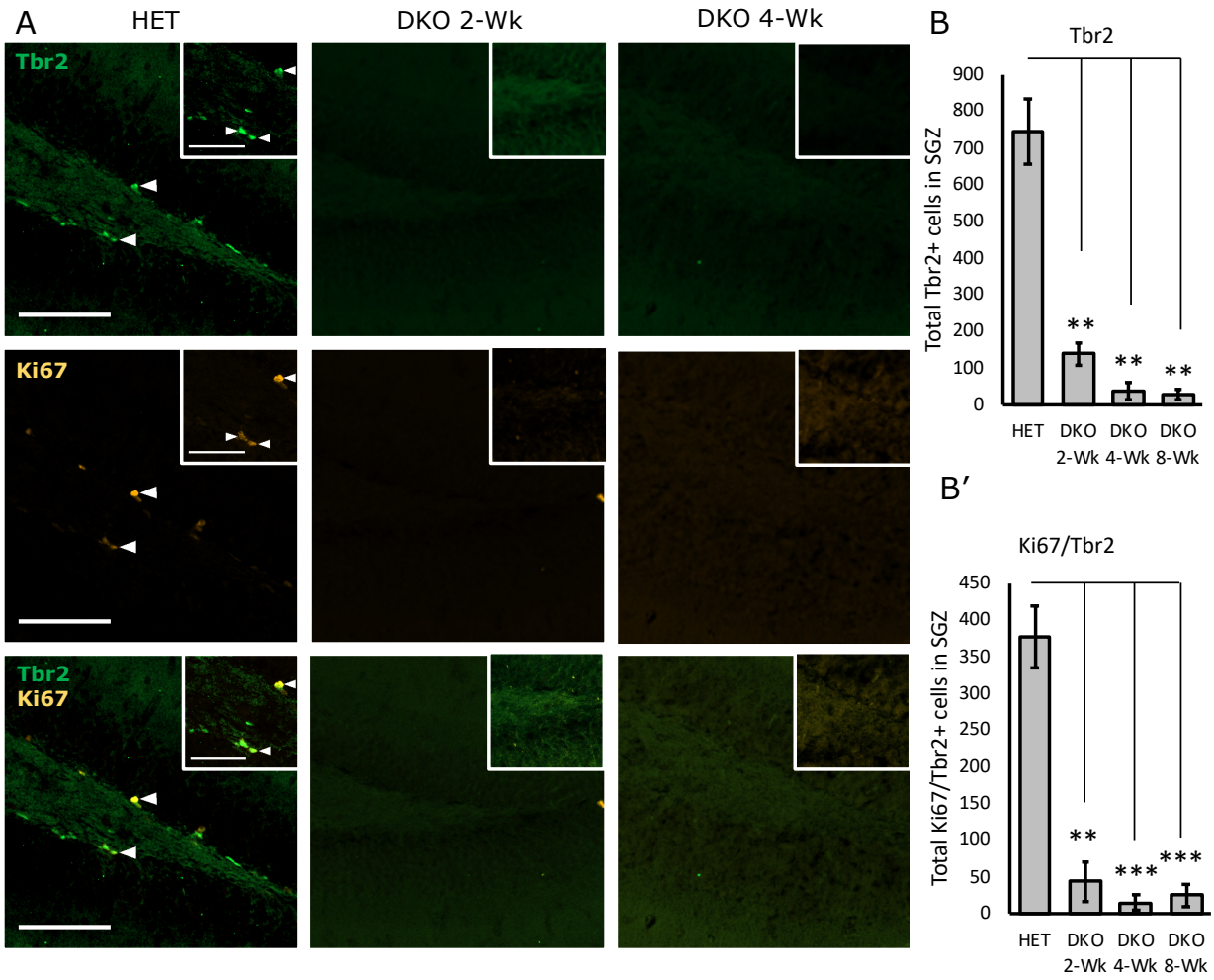


Figure 7. **E2fs1/3 Deletion Reduces the Number of Transit-Amplifying Progenitors In The Subgranular Zone Of The Adult Brain.**
(A) Representative confocal images of total transit-amplifying progenitors (Tbr2+) and their proliferation (Ki67+/Tbr2+) in the adult SGZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Arrowheads show Tbr2+ and Tbr2+/Ki67+ co-localized cells.
(B) Quantification of total transit-amplifying progenitors (Tbr2+) in the adult SGZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Significant reduction in Tbr2+ cells between HET and DKO 2-Wk (**p<0.01), HET and DKO 4-Wk (**p<0.01), HET and DKO 8-Wk (**p<001) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing. **(B')** Quantification of total transit-amplifying progenitors (Ki67+/Tbr2+) in the adult SGZ at either baseline levels (HET), 2-weeks (DKO 2-Wk) or 4-weeks (DKO 4-Wk) after E2Fs1/3 DKO. Significant reduction in Ki67+/Tbr2+ cells between HET and DKO 2-Wk (**p<0.01), HET and DKO 4-Wk (***p<0.001) HET and DKO 8-Wk (**p<001) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing.

3.3 Characterizing the role of E2Fs 1 and 3 in Sox2-cell proliferation and activation

Following our observations of differentiated progenitor loss after E2Fs1/3 DKO, we decided to look at Sox2 populations as a means to assess the activity of undifferentiated precursors, targeting both active and quiescent undifferentiated stem cells. In the SVZ, overall Sox2 population counted along the entirety of the niche was significantly reduced at 2, 4, and 8-weeks ($p<0.05$, $p<0.05$, $p<0.05$ respectively) (Figure 8B) after Tamoxifen administration in DKO mice compared to HET mice, with a 41% decrease in total Sox2 population by 8-weeks. Proliferation of Sox2 cells marked by co-labeled Ki67/Sox2 was also significantly reduced at 2, 4, and 8-weeks ($p<0.01$, $p<0.01$, $p<0.01$ respectively) (Figure 8B') in DKO mice compared to HET mice, with a 90% decrease in total co-labeled Ki67/Sox2 population by 8-weeks. Characterization of this niche led us to conclude that E2Fs impact NSCs at their earlier stage prior to differentiation. We subsequently characterized the behavior of Sox2 cells lining the SGZ of the DG. Overall Sox2 population counted along the entirety of the niche was significantly reduced at 4, and 8-weeks ($p<0.05$, $p<0.05$ respectively) by 20% and 14% respectively but not at 2-weeks, after Tamoxifen administration in DKO mice compared to HET mice, with a 20% decrease in total Sox2 population by 8-weeks (Figure 9B). Additionally, proliferation of SGZ Sox2 population was also significantly reduced as marked by Ki67/Sox2 co-staining ($p<0.01$, $p<0.01$, $p<0.01$ respectively) in DKO mice compared to HET mice, with a 94% decrease in total co-labeled Ki67/Sox2 population by 8-weeks (Figure 9B'). We concluded here that Sox2+ precursor cells lining both niches appear to undergo cell-cycle arrest and are unable to re-enter the cell-cycle in order to

generate new neurons. However, the possibility of these cells entering a state of senescence is also possible.

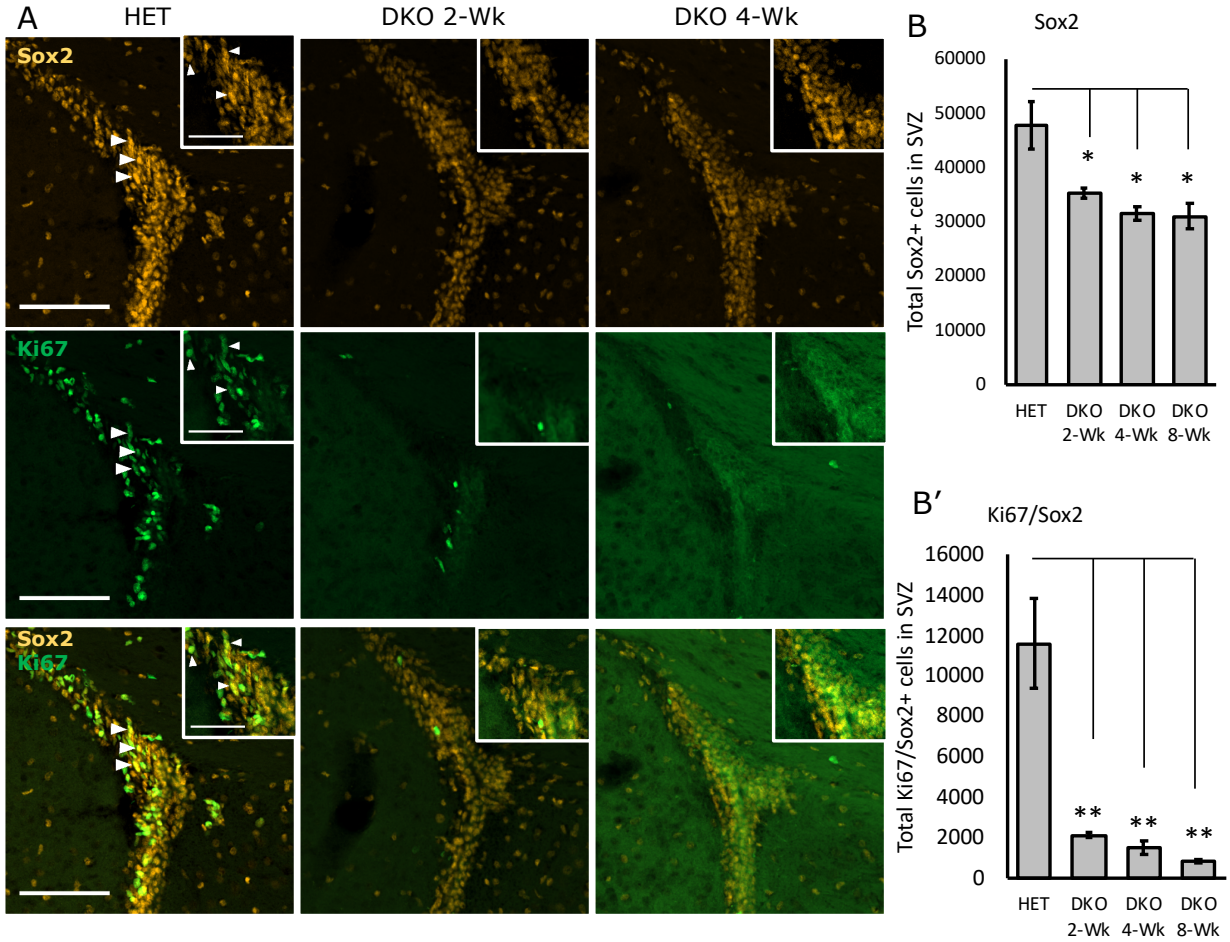


Figure 8. **E2fs1/3 Deletion Reduces the Number of Sox2+ cells In The Subventricular Zone Of The Adult Brain.**
(A) Representative confocal images of total Sox2+ population and their proliferation (Ki67+/Sox2+) in the adult SVZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Arrowheads show Sox2+ and Sox2+/Ki67+ co-localized cells.
(B) Quantification of total Sox2+ population in the adult SVZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk), or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Significant reduction in Sox2+ cells between HET and DKO 2-Wk (*p<0.05), HET and DKO 4-Wk (*p<0.05) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing. **(B')** Quantification of total Ki67+/Sox2+ population in the adult SVZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Significant reduction in Ki67+/Sox2+ cells between HET and DKO 2-Wk (**p<0.01), HET and DKO 4-Wk (**p<0.01), HET and DKO 8-Wk (**p<0.01) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing.

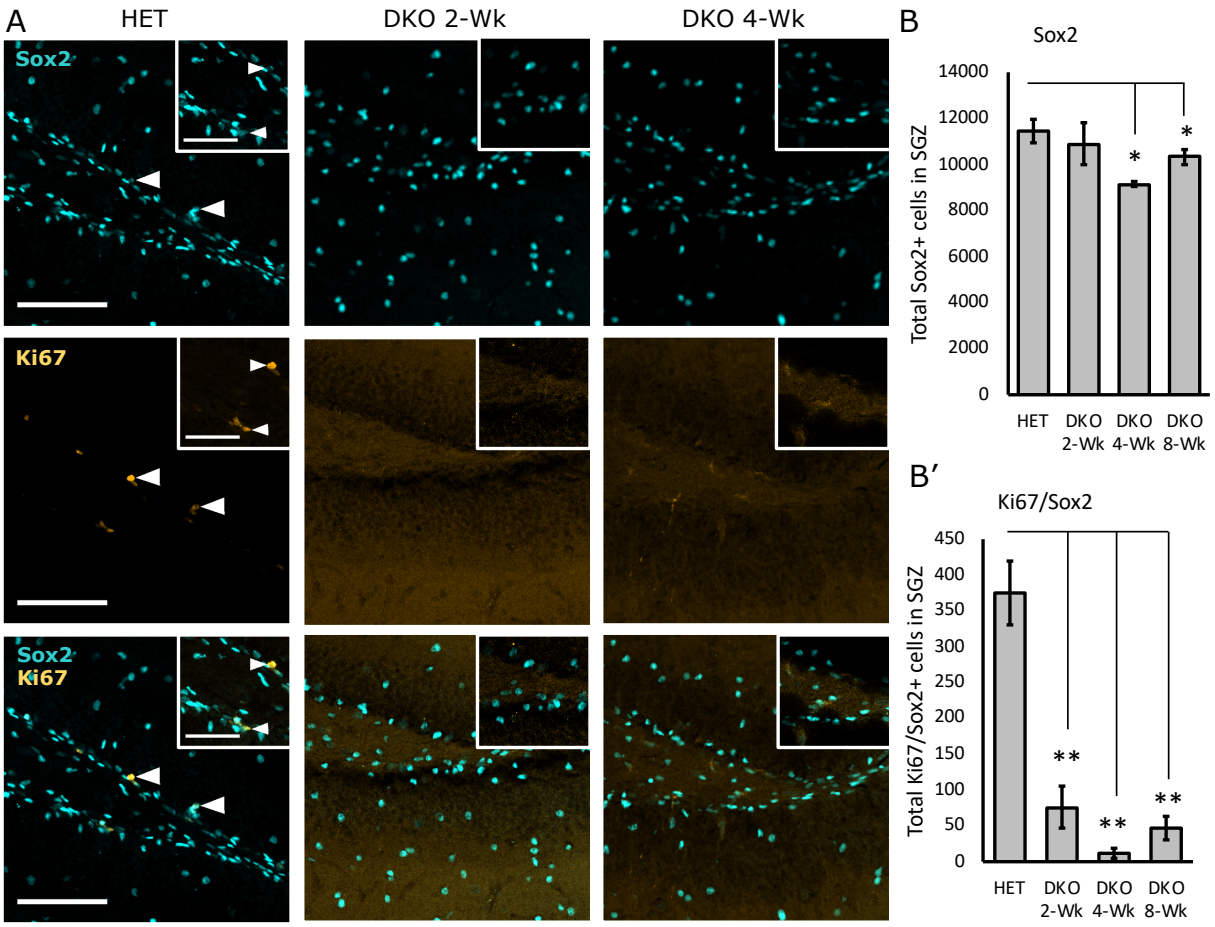


Figure 9. **E2fs1/3 Deletion Reduces the Number of Sox2+ cells In The Subgranular Zone Of The Adult Brain.**
(A) Representative confocal images of total Sox2+ population and their proliferation (Ki67+/Sox2+) in the adult SGZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Arrowheads show Sox2+ and Sox2+/Ki67+ co-localized cells.
(B) Quantification of total Sox2+ population in the adult SGZ at either baseline levels (HET), 2-weeks (DKO 2-Wk), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Significant reduction in Sox2+ cells HET and DKO 4-Wk (*p<0.05), HET and DKO 8-Wk (*0<0.05) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing. **(B')** Quantification of total proliferating Ki67+/Sox2+ population in the adult SGZ at either baseline levels (HET), 2-weeks (DKO 2-Wk) or 4-weeks (DKO 4-Wk) after E2Fs1/3 DKO. Significant reduction in Ki67+/Sox2+ cells between HET and DKO 2-Wk (**p<0.01), HET and DKO 4-Wk (**p<0.01), HET and DKO 8-Wk (**p<0.01) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing.

3.4 Elucidating the effects of E2Fs 1 and 3 deletion on quiescence and activation in the SVZ

After characterizing the Sox2 population in the SVZ and noting a significant reduction in the overall population of Sox2+ cells, we decided to look at the ability of NSCs to enter the cell-cycle and whether there is reduced ability to activate in those cells. Specifically, we reasoned that due to the lack of cell-cycle among the Sox2 cells there could be a defect in the ability of NSCs to exit quiescence. Following our discovery that proliferating progenitors are absent from the neurogenic niches, along with a distinct reduction in overall stem cell numbers marked by Sox2, we asked whether E2Fs1/3 are required for quiescent stem cell activation for initiation of neurogenesis. To answer this question, we utilized a novel panel of cell markers with the intention to mark for quiescent NSCs (qNSCs) and a stage that is readily primed for proliferation and differentiation termed active NSCs (aNSCs) (Codega et al., 2014). Briefly, a co-label stain of Glial-Fibrillary Acidic Protein (GFAP) and Prominin-1 (CD133) in the absence of Endothelial Growth Factor Receptor (EGFR) marks for qNSCs, whereas a triple-label of GFAP/CD133/EGFR marks for aNSCs in the SVZ. After staining for these markers, we noticed a significant decrease in the percentage of EGFR+ cells that are co-labeled for GFAP/CD133 4- and 8-weeks after Tamoxifen administration (11% and 15% respectively) and to the mice when DKO-mice are compared to HET (*$p<0.05$, *$p<0.05$ respectively) (Figure 10). Based on the finding that EGFR+ active NSCs are reduced following E2Fs1/3 deletion, we concluded that the ability of NSCs to enter their active state is severely impaired, and these cells may be stuck in quiescent state with a reduction

of 15% in the proportional number of EGFR+ active cells by 8-weeks after acute knockout.

To further confirm the result, we decided on another panel of markers to target cell-cycle exit rather than quiescence, since quiescence is a form of cell-cycle withdrawal (Blagosklonny, 2011). The chosen panel employs Cyclin-Dependent Kinase 2A (p16), a cell-cycle exit marker, and Sox2, with the presence of co-labeled p16/Sox2 denoting neural precursors that are in a cell-cycle exit state yet still possess stemness qualities to them. The resultant characterization indicates greater cell-cycle exit among the Sox2 population marked by significantly increased Sox2/p16 co-labelling 4-weeks after DKO compared to HETs ($*p<0.05$) (Figure 11). The results showing increased cell-cycle exit among SVZ NSCs in addition to reduced activity marker EGFR+ suggest that E2Fs1/3 are crucial for activation of NSCs from quiescence and initiation of neurogenesis in the adult brain.

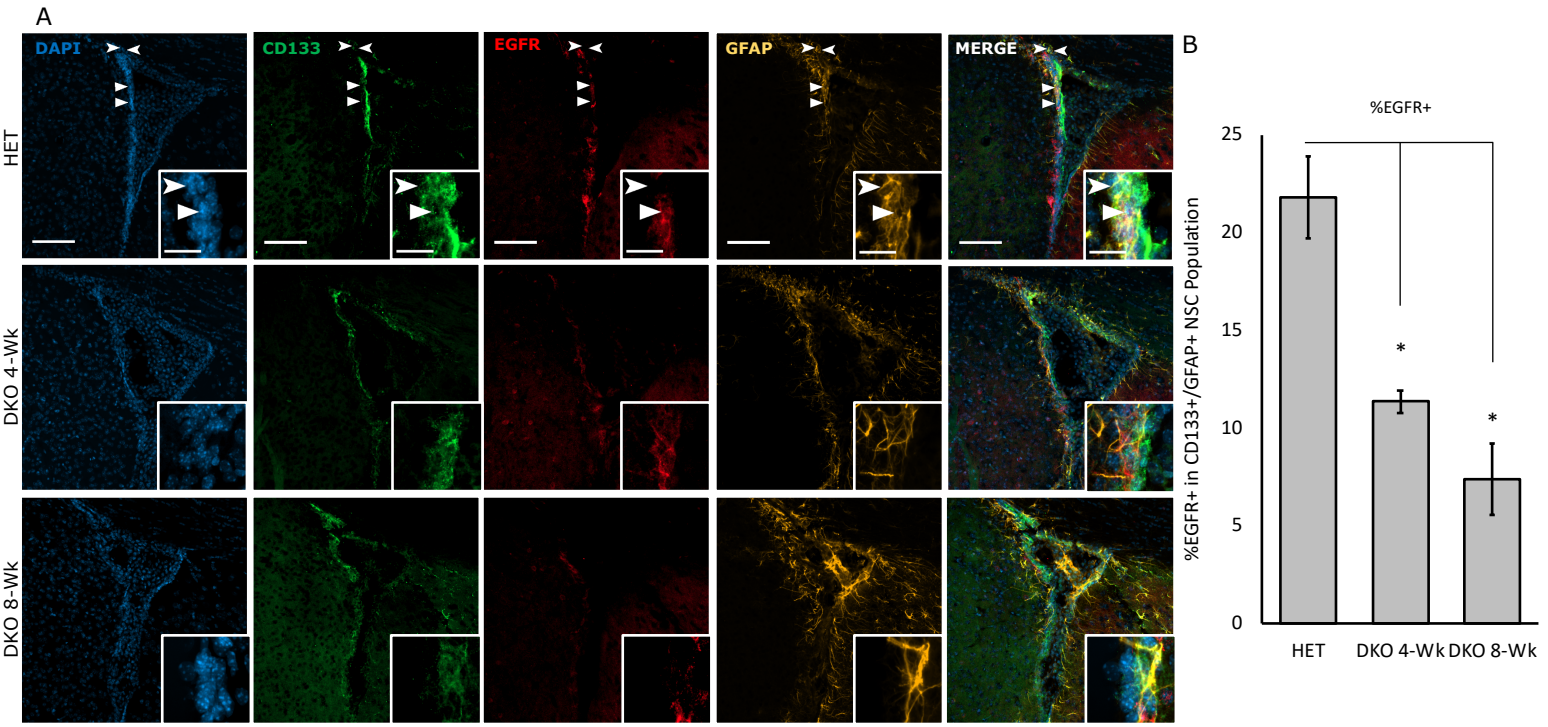


Figure 10. **E2fs1/3 Deletion Reduces the Activation of NSCs In The Subventricular Zone Of The Adult Brain.**
(A) Representative confocal images of nuclear staining (DAPI+), stem and ependymal marker (CD133+), activation (EGFR+), stem and astrocyte marker (GFAP+), in the adult SVZ (dorsolateral region) at either baseline levels (HET), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Notched arrowheads show quiescent stem cells (DAPI+/CD133+/GFAP+/EGFR-). Filled arrowheads show active stem cells (DAPI+/CD133+/GFAP+/EGFR+).
(B) Quantification of active NSCs (DAPI+/CD133+/GFAP+/EGFR+) as a fraction of total NSCs (DAPI+/CD133+/GFAP+) in the adult SVZ at either baseline levels (HET), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Significant reduction in percent active NSCs between HET and DKO 4-Wk (*$p<0.05$) and DKO 8-Wk (*$p<0.05$) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing.

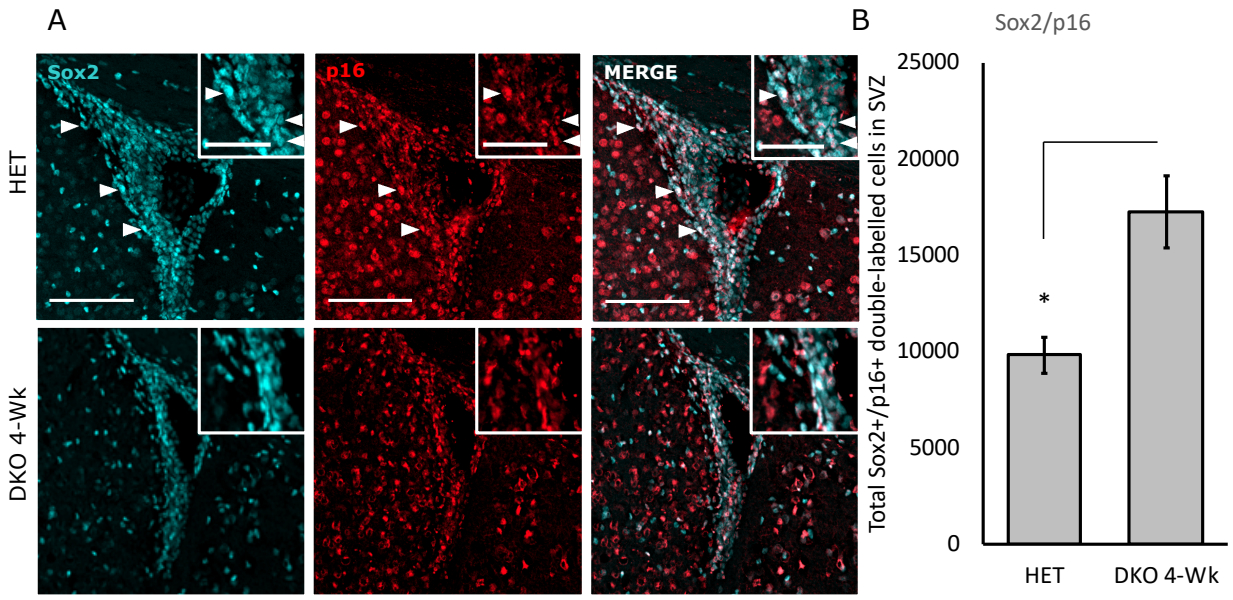


Figure 11. **E2fs1/3 Deletion Increases Cell-Cycle Exit Among NPCs In The Subventricular Zone Of The Adult Brain.**
(A) Representative confocal images of NPCs (Sox2+) and cell-cycle exit gene (p16+) in the adult SVZ at either baseline levels (HET) or 4-weeks (DKO 4-Wk) after E2Fs1/3 DKO. Arrowheads show NPCs that have withdrawn from the cell-cycle (Sox2+/p16+).
(B) Quantification of double-labelled Sox2+/p16+ cells in the adult SVZ at either baseline levels (HET) or 4-weeks (DKO 4-Wk) after E2Fs1/3 DKO. Significant increase in total Sox2+/p16+ between HET and DKO 4-Wk (*$p<0.05$) in a Student's t-test (n=3).

3.5 Elucidating the effects of E2Fs 1 and 3 deletion on quiescence and activation in the SGZ

Following our characterization of the SVZ, we decided to explore whether a similar defect in NSC activation could be seen in the SGZ. In this characterization, we utilized a novel marker panel of factors discovered through Single-Cell RNA-Seq on sorted SGZ cells in combination with pseudotime correction to clarify targets that mark for stages of quiescence and activation (Shin et al., 2015). In SGZ NSC transcriptome analysis, Aldoc protein is present during the quiescent stage of the cell, and is rapidly downregulated following activation. We choose Aldoc to mark for qNSCs in the SGZ, whereas its absence in a Nestin+ or GFAP+ cell denotes and exit from quiescence into an aNSC sub-type. For this analysis, we used Aldolase-C (Aldoc) together with Sox2 and GFAP to mark quiescent SGZ cells, with Aldoc being highly upregulated in qNSCs and immediately downregulated following activation (Shin et al., 2015). Active NSCs were marked with Sox2 and Nestin in the absence of Aldoc to capture the population of active precursors (Codega et al., 2014; Lugert et al., 2010; Shin et al., 2015). Percentage of total Sox2/GFAP population co-labeled with Aldoc was significantly increased 8-weeks following Tamoxifen by 19% ($p<0.001$) (Figure 12), suggesting a higher proportion of SGZ NSCs are in a qNSC state. Total Sox2/Nestin population was reduced 8-weeks following Tamoxifen administration ($p<0.05$) as well as the total Sox2 population ($p<0.05$), suggesting that there may be a defect in NSC activation, and total number NSCs is down along the SGZ (Figure 13). To confirm whether activation is indeed downregulated following DKO, we looked at pro-neural activation protein Ascl1 in the SGZ (Urbán et al., 2016). Total population count of Ascl1 was significantly reduced 4-

and 8-weeks following Tamoxifen administration by 93% and 98% respectively ($p<0.001$) (Figure 14B), and proliferation assessed by Ascl1/Ki67 co-labeling also shows a significant reduction in total population count by 98% ($p<0.001$) (Figure 14B'). We conclude there may be a defect in inability of NSCs to exit quiescence in the SGZ after knockout, similar to SVZ, lending further evidence to our hypothesis that E2Fs1/3 are crucial for NSC activation.

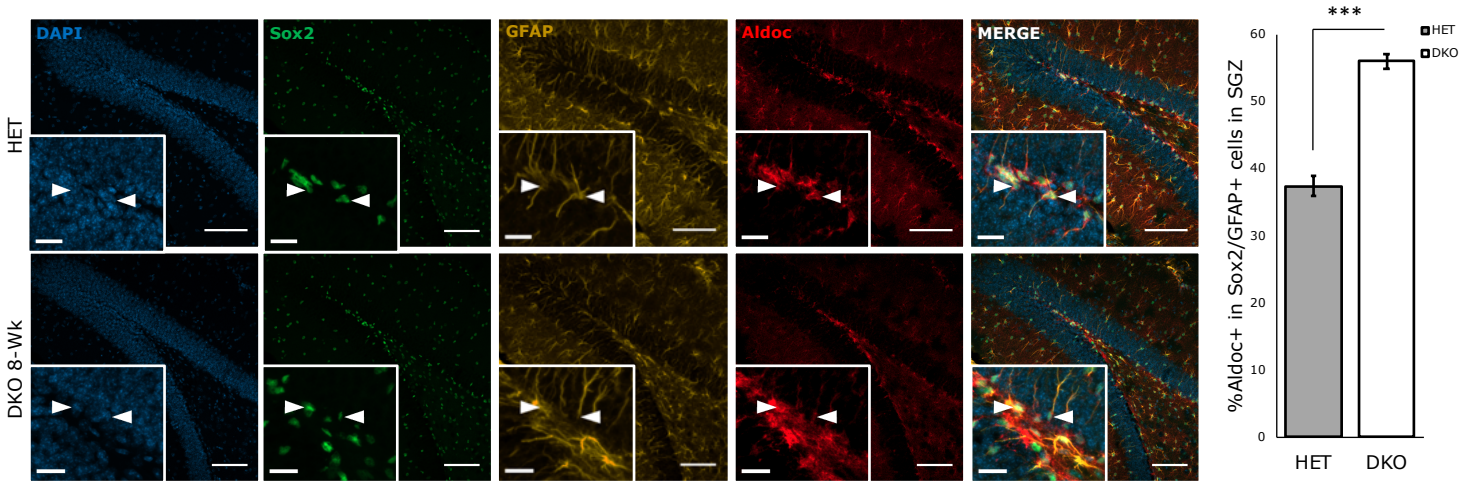


Figure 12. **E2fs1/3 Deletion Increases the Population of Aldoc+ qNSCs in the Subgranular Zone Of The Adult Brain.**
(A) Representative confocal images of nuclear staining (DAPI+), quiescence (Aldoc+) glial morphology (GFAP+), NPC marker (Sox2+), in the adult SGZ at either baseline levels (HET), 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Filled arrowheads show quiescent NSCs (DAPI+/Aldoc+/Sox2+/GFAP+).
(B) Quantification of percent quiescence (%Aldoc+) in adult neural stem cells (Sox2+/GFAP+) in the adult SGZ at either baseline levels (HET) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Significant reduction in total Sox2+ between HET and DKO 8-Wk (***$p<0.0001$), significant increase in %Sox2+/GFAP+/Aldoc+ in DKO 8-Wk (*$p<0.05$) in a Student's t-test (n=3).

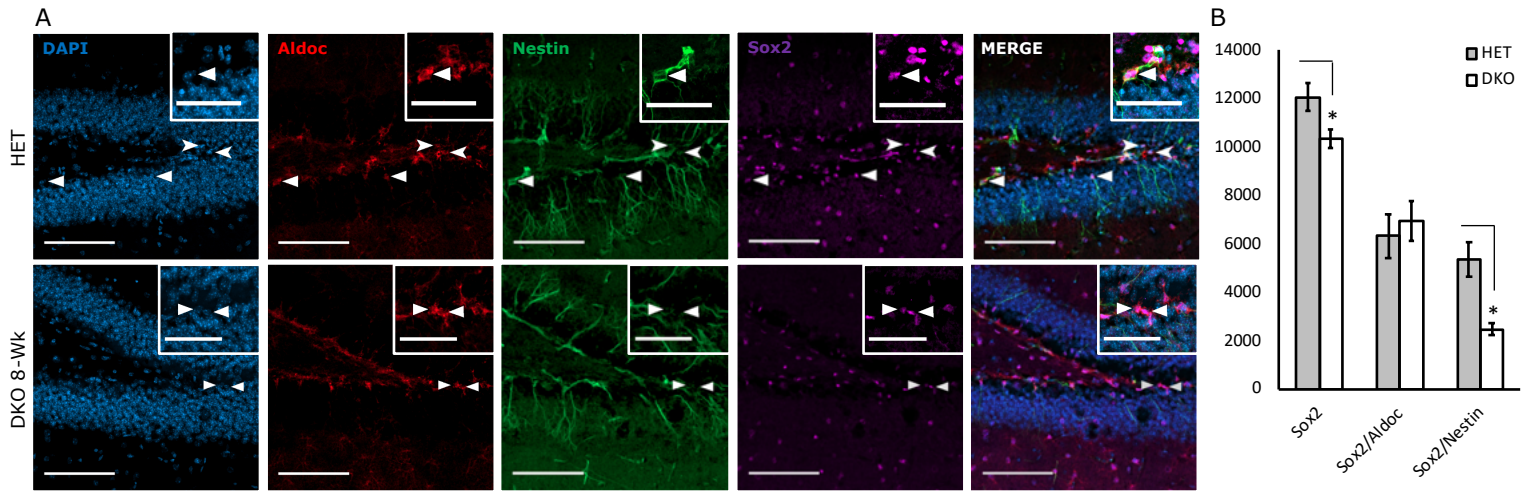


Figure 13. **E2fs1/3 Deletion Reduces the Number of Nestin+ NSCs in the Subgranular Zone Of The Adult Brain.**
(A) Representative confocal images of nuclear staining (DAPI+), quiescence (Aldoc+) stem and activity marker (Nestin+), stem cell marker(Sox2+), in the adult SGZ at either baseline levels (HET), 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Notched arrowheads show quiescent NSC (DAPI+/Aldoc+/Sox2+/Nestin-). Filled arrowheads show active NSC (DAPI+/Aldoc+/Sox2+/Nestin+).
(B) Quantification of total NPCs (Sox2+), qNSCs (Sox2+/Aldoc+), and aNSCs (Sox2+/Nestin+) in the adult SGZ at either baseline levels (HET) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Significant reduction in total Sox2+ between HET and DKO 8-Wk (*$p<0.05$), significant reduction in Sox2+/Nestin+ and DKO 8-Wk (*$p<0.05$) in a series of Student's t-tests (n=3).

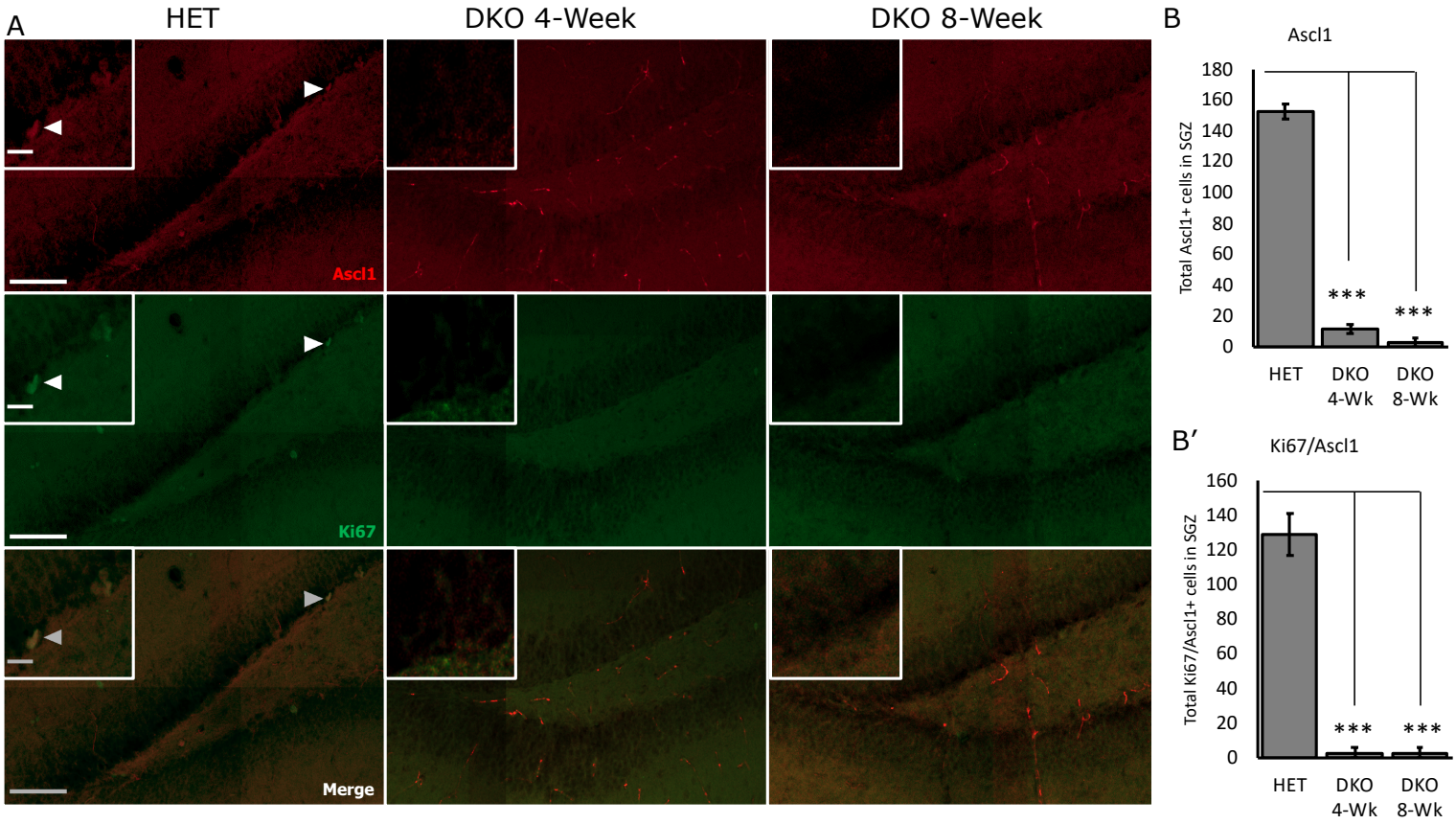


Figure 14. **E2fs1/3 Deletion Reduces the Number of Pro-Neural cells in the Subgranular Zone.**
(A) Representative confocal images of total pro-neural cells (Ascl1+) and their proliferation (Ki67+/Ascl1+) in the adult SGZ at either baseline levels (HET), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Arrowheads show Ascl1+ and Ascl1+/Ki67+ co-localized cells. **(B)** Quantification of total transit-amplifying progenitors (Ascl1+) in the adult SGZ at either baseline levels (HET), 4-weeks (DKO 4-Wk) or 8-weeks (DKO 8-Wk) after E2Fs1/3 DKO. Significant reduction in Ascl1+ cells between HET and DKO 4-Wk (***p<0.001), HET and DKO 8-Wk (***p<0.001) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing. **(B')** Quantification of total transit-amplifying progenitors (Ki67+/Ascl1+) in the adult SGZ at either baseline levels (HET), 4-weeks (DKO 2-Wk) or 8-weeks (DKO 4-Wk) after E2Fs1/3 DKO. Significant reduction in Ki67+/Tbr2+ cells between HET and DKO 4-Wk (***p<0.001) HET and DKO 8-Wk (***p<0.001) in a 1-Way independent ANOVA (n=3) with Tukey's post-hoc testing.

3.6 Transcript-level analysis of a potential mechanism for E2F activation of NSCs

Following characterization of NSC behavior in the niches after knockout, our group set out for follow up on these initial studies (work done by Daniel O'Neil, Bensun Fong) by probing known targets of E2Fs to gather increasing support for our hypothesis, and perform a transcript-wide analysis via RNA-Sequencing to study gene networks specific to quiescence and activation. Our group sorted cells from embryonic cortices that are either knockouts or heterozygous-controls for E2Fs1/3. Briefly, pregnant mothers were given a single dose of Tamoxifen at E15.5 and the pups were harvested three days after at E18.5. After dissecting and digesting cells lining the ventral VZ, our group sorted CD15+ cells and extracted RNA for qPCR analysis. Our goal here was to target the NSC population as early as its born at approximately E14.5 and probe targets of activation and survival Ascl1, EGFR and Sirtuin-1 (SIRT1) (Fuentealba et al., 2015; Furutachi et al., 2015). Results show mRNA levels for EGFR, SIRT1, Ascl1 are significantly reduced following a single Tamoxifen infusion in embryonic cortices of E2Fs1/3 DKO mice compared to HET ($p<0.05$, $p<0.01$, $p<0.001$ respectively) (Figure 15). In addition, our group confirmed our knockout efficiency in the embryonic model by probing for E2F1 and E2F3 with both significantly downregulated at the mRNA-level following a single Tamoxifen treatment ($p<0.001$, $p<0.01$ respectively) (Figure 16).

Based on the previous qPCR analysis, our group (Work done by Daniel O'Neil, Bensun Fong) performed RNA-Sequencing on knockout cells that we dissected and homogenized from the SVZ of adult mice based on our canonical paradigm. In order to gauge whether there is an impact on NSC quiescence and activation our group (Work done by Daniel O'Neil, Bensun Fong) performed initial network analysis where we

overlaid our RNA-Seq data with published data of genes and networks unique to either qNSCs or aNSCs (Codega et al., 2014). The bioinformatic overlay reveals that 457 unique qNSC signatures are upregulated, whereas only 16 signatures are downregulated. Furthermore, 541 unique aNSC signatures are downregulated, while only 4 are upregulated (Figure 17). The result demonstrates an increase in the potential unique regulatory gene networks responsible for maintaining cells in quiescence while a reduction in such genes responsible for activation of cells, suggesting that activator E2Fs1/3 may be responsible for regulating a vast and complex array of mechanisms that promote NSC exit from quiescence.

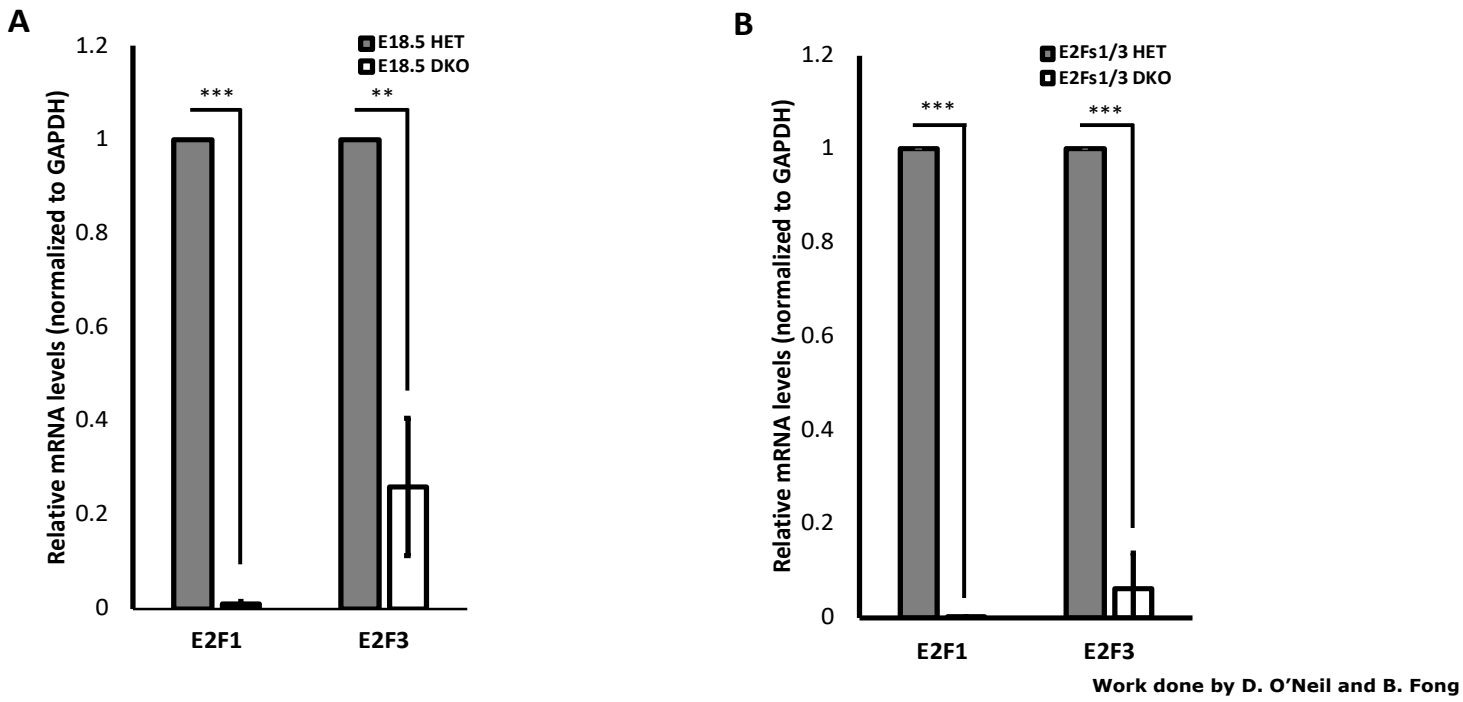


Figure 15. **E2fs1/3 mutation is present throughout the developing cortex and the adult cortex following Tamoxifen treatment.** **(A)** Quantitate real-time PCR data of mRNA extracted from cells dissected from embryonic E18.5 ventral cortex of mice of HET and E2Fs1/3 DKO mice. Significant reduction in E2F1 (***p<0.001), E2F3 (**p<0.01) mRNA levels after E2Fs1/3 DKO in a Student's t-test (n=3). **(B)** Quantitate real-time PCR data of mRNA extracted from cells dissected from adult SVZ of mice of HET and E2Fs1/3 DKO mice. Significant reduction in E2F1 (***p<0.001), E2F3 (***p<0.01) mRNA levels after E2Fs1/3 DKO in a Student's t-test (n=3).

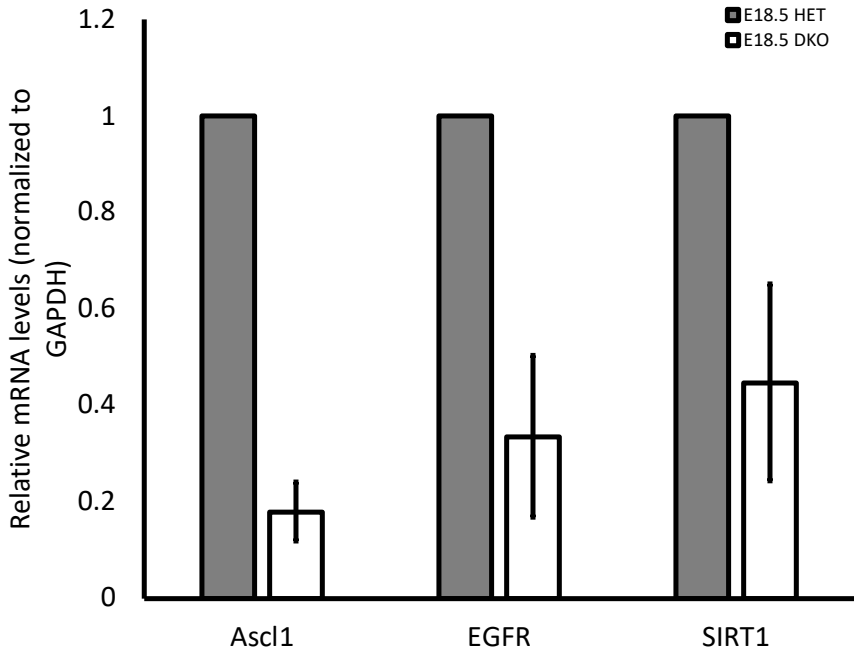


Figure 16. **E2fs1/3 Deletion Impairs Activation and Pro-Neural Lineage in the Developing Embryo Cortex.**
(A) Quantitate real-time PCR data of mRNA extracted from cells dissected from embryonic E18.5 ventral cortex of mice of HET and E2Fs1/3 DKO mice. Significant reduction in Ascl1 (***$p<0.001$), EGFR (**$p<0.01$), and SIRT1 (*$p<0.05$) mRNA levels after E2Fs1/3 DKO in a Student's t-test (n=3).

Figure 17. **Downstream effects of E2Fs1/3 result in stem cell bearing increased transcript similarity to quiescent cells and reduced transcript similarity to active cells.** The library generated in this paper was then compared and overlaid with the library published in Codega, 2014 to assess whether stem cells behave with quiescent or active characteristics following E2Fs1/3 DKO.
(A) Chart demonstrating stem cells upregulation of transcripts unique to qNSCs following E2Fs1/3 DKO from sorted SVZ cells. Cells were sorted from YFP+ Rosa26-reporter single-cell population and harvested for RNA sent for sequencing. Cell sorting and data-analysis was done by Daniel O'Neil and Bensun Fong.
(B) Chart demonstrating stem cells downregulation of transcripts unique to aNSCs following E2Fs1/3 DKO from sorted SVZ cells. Cells were sorted from YFP+ Rosa26-reporter single-cell population and harvested for RNA sent for sequencing. Cell sorting and data-analysis was done by Daniel O'Neil and Bensun Fong.

SUMMARY OF RESULTS

The work presented in this thesis determines that E2Fs 1 and 3 possess a potential role in the activation of NSCs in the niches of the adult brain. Animals lacking both E2F1 and E2F3 demonstrated a significant downturn in the neurogenic capacity of their NSC niches. Particularly, E2Fs1 and 3 may work together to target the transition of qNSC to aNSC shown using a novel marker panel that seeks to elucidate newly discovered sub-types of neural NSCs. Therefore, E2Fs1/3 may have a significant regulatory role in neurogenesis and can be potential targets for tissue regeneration following brain insult in the future. Throughout the characterization and analysis conducted in this paper, we have obtained some novel discoveries about the role of E2Fs in the regulation of adult neurogenesis:

1) E2Fs1/3 are required for generation of newborn neurons and proliferation of all precursor types is significantly reduced following E2Fs1/3 DKO. 2) Ascl1, a pro-neural activation factor, appears to be reduced following DKO suggesting it may be downstream of E2Fs1/3. 3) E2Fs1/3 are required to transition qNSC sub-types into aNSC sub-types of niche NSCs.

Along with a significant reduction in adult neurogenesis, we have demonstrated that E2Fs1/3 affect newly discovered NSC subtypes. Interestingly, this effect appears to be similar in both the SVZ and SGZ, suggesting that E2Fs1/3 may be good targets for regulation of neurogenesis not only for tissue regeneration but also for potential regulation of mood and cognition. Taken together, the results support the hypothesis that **E2F1 and E2F3 are required for NSC exit from quiescence and pro-neural activation.**

DISCUSSION

4.1 Activator E2Fs1/3 are required for neurogenesis in both SVZ and SGZ niches

Our results show there is a defect in generation of newborn neurons marked by DCX following DKO. In addition to this, proliferation among the DCX population is also reduced marked by Ki67, suggesting that fewer precursors are able to proliferate and differentiate into newborn neuron-type cells (Alvarez-Buylla and García-Verdugo, 2002). This data is consistent with single-knockout studies of E2F1 and E2F3 demonstrating that the transcription factors affect more mature, late-stage precursor cells in the SVZ and SGZ niches (Cooper-Kuhn et al., 2002; Julian et al., 2013; McClellan et al., 2007). Although single-knockout of either E2F1 and E2F3 reduces proliferation and neurogenesis by precursors in the niches, neurogenesis does not appear to be completely halted (Cooper-Kuhn et al., 2002; McClellan et al., 2007). Therefore, it is possible that E2F1 and E2F3 have compensatory mechanisms when lacking one or the other in adult NSCs that still allow certain precursors to proliferate and differentiate albeit at a reduced capacity (Chen et al., 2009b; Kong et al., 2007). Further compounded by our results showing an almost complete arrest of neurogenesis after E2Fs1/3 DKO, it appears that both transcription factors play a key role together in the neurogenic capacity of NSCs and further differentiated precursors. We observed the arrest of neurogenesis by assessing several precursor cell populations via markers DCX, Tbr2, Ascl1, and Sox2 to show that there is a disruption in proliferation and differentiation from undifferentiated NSCs to more mature cell-types. This suggests that E2F requirement for neurogenesis may begin at the earliest, most primitive stage of the NSC lineage.

4.2 Activator E2Fs1/3 reduce levels of pro-neural factor Ascl1 in neurogenic precursors

Ascl1 has emerged as a pro-neurogenic factor important for guiding the lineage of NSCs towards a neuronal cell-type fate as well as priming cells for activation from a state of quiescence (Castro et al., 2011; Kim et al., 2011; Urbán et al., 2016; Vasconcelos and Castro, 2014). Pro-neural differentiation factor Ascl1 may have the capacity to not only potentially wake dormant NSCs and force them into the cell-cycle, it may also divert the fate of those cells to a neural one. As stated, pro-quiescence and cell-cycle proteins could be the major suppressant and indeed a barrier to Ascl1 activity. Therefore, establishing a link between cell-cycle proteins, such as the E2F-family of transcription factors, to pro-neural activation factors like Ascl1 could potentially provide meaningful conclusions about the regulation of quiescent NSC transitions into a pro-neural active state.

We asked in our study if E2Fs could potentially be required for pro-neural activation. Based on our results showing that all precursor populations post activation, such as Tbr2 and DCX, are almost completely ablated while the Sox2 population only gradually decreases over time, we surmised there may be a lack of activation of NSCs towards a neural fate. After staining for aforementioned pro-neural marker and factor Ascl1 in both niches and noting a significant lack of the maker in both niches, we concluded that a lack of E2Fs results in reduced neurogenesis, and we speculate that this may happen through loss of Ascl1, leading to impaired pro-neural activation in the niche. We confirmed this through qPCR analysis of RNA isolated from CD15+ early-born adult NSCs in the embryonic cortex, showing that Ascl1 mRNA is downregulated along with other critical targets such as neuroblast pro-survival factor SIRT1 (Figure 14) (Fuentealba et al., 2015; Furutachi et al., 2015; Pfister et al., 2008).

We are uncertain whether E2Fs directly regulate Ascl1 to induce pro-neural activation in the niches, which is a potential future avenue to explore the mechanism by which E2Fs control neurogenesis in the niches. However, ChIP-on-Chip analysis from our lab shows that E2F3 possesses great binding affinity for the Ascl1 promoter region, suggesting these factors may be intertwined in a complex relationship that can influence NSCs to activate towards a neural fate (Julian et al., 2013). It is not surprising to find that overexpression of Ascl1 in proliferating precursors can change their glial and astrocytic fate to a pro-neural one, even in disease models, and we believe that E2Fs can modulate this interaction further (Faiz et al., 2015).

4.3 Activator E2Fs1/3 reduce the ability of qNSCs to transition to an aNSC sub-type

As mentioned, while the proliferative precursor populations are reduced, the quiescent Sox2 population was marginally reduced when compared (Figure 6, 7), suggesting that E2Fs may also regulate NSC activation towards self-renewal, prompting us to look at the most primitive state of NSCs. Recent advances in whole-genome transcriptomics revealed the existence of several NSC sub-types which were previously difficult to identify. Adult NSCs lining the niches are found in either a quiescent state (qNSC) or an active state (aNSC), and unlike differentiation into a more specified precursor cell, these two states are reversible where a cell may exit and enter quiescence multiple times to divide asymmetrically (Codega et al., 2014; Llorens-Bobadilla et al., 2015; Shin et al., 2015).

We began investigating the behavior of these NSC sub-types following our E2Fs1/3 DKO by characterizing the niches using immunofluorescence. We discovered that using a unique panel of markers for the SVZ and the SGZ allowed us to elucidate a

defect in the ability of qNSCs to transition to an active state. In the SVZ, qNSCs marked by CD133/GFAP and the absence of EGFR appeared to be in higher proportion when compared to aNSCs marked with triple co-localization of CD133/GFAP/EGFR. Additionally, cell-cycle exit marker p16 staining revealed that the majority of Sox2 NSCs in the SVZ are found in a state of cell-cycle exit. Taken together, we conclude that a lack of E2Fs1/3 prevents NSCs from exiting quiescence and in fact creates a barrier between G0 to S-phase transitions, highlighting the crucial need for activator E2Fs in cellular activation.

In the SGZ, we utilized another novel panel of Aldolase-C (Aldoc) marker as the predominant factor expressed in dentate NSC quiescence and is the first factor to be downregulated following activation (Shin et al., 2015). Using a combination of Aldoc and Nestin as markers of quiescence and activation respectively we were able to discern qNSCs from aNSCs in the SGZ (Lugert et al., 2010). With a higher population of Aldoc cells in the SGZ in the face of an overall decline in Sox2/Nestin co-labeled cells, we discovered that activator-E2Fs1/3 deletion yields greater quiescence among NSCs in the SGZ as well (Figure 10, 11).

Lastly, RNA-Sequencing was performed on RNA from recombined cells dissected from the SVZ by collecting YFP-reporter cells (O'Neil, Fong, Yakuvovich, *Unpublished*). We then combined our transcriptome with another published transcriptome encompassing unique targets and networks to either qNSCs or aNSCs (Codega et al., 2014). The resultant overlay showed that after E2Fs1/3 DKO, the transcriptome of SVZ NSCs is more akin to qNSCs than aNSCs by way of upregulating factors unique to qNSCs and downregulating factors unique to aNSCs. Based on this and previous results,

we hypothesize that activator E2Fs1/3 are crucial regulators of NSC quiescence and are mechanistically required for qNSCs to transition to aNSCs and engage in neurogenesis.

4.4 Alternative Explanations

Activator E2Fs1/3 are required for cell-cycle entry and progression (Giacinti and Giordano, 2006; Lukas et al., 1997). An overwhelming abundance of cell-cycle exit protein p16 was observed along the SVZ niche following E2Fs1/3 DKO suggesting that without these transcription factors, cell-cycle entry has stunted. However, it is possible that deletion of activator E2Fs1/3 shifts a balance of transcription factors to favour quiescence over activation, rather than preventing activation. As E2Fs1/3 levels rise and fall in cells throughout G1 -> S-Phase transitions, downstream target expression switches from a network of quiescent cells to active cells and vice versa. We propose that rather than controlling a single downstream network of activation genes, E2F1/3 may modulate two separate networks that are in opposition to each other.

Inducing E2Fs1/3 deletion may affect the overall activity of the Rb/E2F pathway. In turn, this may cause a cascade that affects numerous cell-types and precursors in the niches. It is pertinent to not discount such potential side-effects of E2Fs1/3 knockout. Deletion of E2Fs1/3 may results in impaired regulation of the upstream CDK/Cyclin pathway. Misregulation of the Rb/E2f pathway may result in a cascade of improper CDK/Cyclin-activity yielding either aberrant over- or under-activation of specific cell-types. It is possible that in our model, knocking out E2Fs1/3 results in a signalling cascade that prevents over-activation of NSCs by affecting upstream CDK/Cyclin activity and subsequent Rb- and Rb-family protein activity. We hypothesize that this cascade could potentially be responsible for the phenotype seen in this paper. For example, it has

been established that CDK-mediated regulation of Sox2 protein is crucial for induction of pluripotency and pro-neural activation of NSCs (Lim et al., 2017; Ouyang et al., 2015). Knockout and overexpression assays of E2Fs1/3 combined with chromatin immunoprecipitation and western blotting could potentially reveal the effects of E2Fs1/3 deletion on the activity of CDKs/Cyclins. Additionally, characterization of NSCs following selective deletion of some CDKs may reveal a similar phenotype to knocking out E2Fs1/3, suggesting that it is the upstream regulation by CDKs that may be responsible for the aberrant neurogenesis phenotype we see in this paper.

4.5 Future Directions

Healthy activation of NSCs is crucial for adult neurogenesis and the modulation of behavior and cognition (Aimone et al., 2014; Becker and Wojtowicz, 2007; Mak and Weiss, 2010). Additionally, activation and survival of NSCs could play a key role in the recovery of brain tissue from diseases such as Alzheimer's, Parkinson's, and stroke in the future. We have unraveled a novel role for E2Fs1/3 in the regulation of adult neurogenesis. Specifically, we have shown that E2Fs1/3 have a key regulatory role in the activation of NSCs and exit from quiescence. However, the specific mechanism and pathway by which this happens remains to be discovered and characterized.

Ascl1 has been demonstrated to be key in the pro-neural fate decision and activation of adult NSCs in both adult niches (Kim et al., 2011; Urbán et al., 2016; Vasconcelos and Castro, 2014). Previous data from ChIP-on-Chip experiments in our lab shows E2F3 has binding affinity for Ascl1 promoter region (Julian et al., 2013), in addition to qPCR experiments in this thesis demonstrating loss of Ascl1 mRNA presence following E2Fs1/3 DKO in adult NSCs shortly after their formation during

embryogenesis. We believe there may be a key regulatory role for activator E2Fs1/3 on Ascl1 gene expression. More specifically, we believe E2Fs1/3 regulate Ascl1 transcription levels in adult NSCs to enact pro-neural exit from quiescence when various extracellular and intracellular signals affect CDK-mediated activation of the Rb/E2F pathway. Ascl1 presents an exciting and novel target for modulation of NSC and transit-amplifying progenitor fate after disease (Faiz et al., 2015). Elucidating the mechanism by which E2Fs1/3 potentially regulates downstream Ascl1 may hold the key for qNSC transition into aNSC with the ability to prime NSCs towards a neural fate, a condition which can prove useful in tissue regeneration following disease. The utility of chromatin immunoprecipitation and luciferase assays may help to reveal direct regulatory role for E2Fs1/3 on Ascl1 transcript levels. Specifically, chromatin immunoprecipitation (ChIP) experiments showing E2Fs1/3 binds Ascl1 promoter could provide such evidence. Additionally reduced luciferase activity bound to an Ascl1 promoter construct following overexpression of E2F1 or E2F3 in culture could indicate downstream regulation of E2Fs1/3 on Ascl1. This would set E2Fs1/3 as potential targets for modulation of crucial pro-neural protein Ascl1. Following this, lentiviral driven-overexpression of either E2F1 or E2F3 in the niches after a prolonged DKO period acting as a rescue will potentially demonstrate whether Ascl1 is turned on with a proper set of markers for characterization. Additionally, Ascl1 lentiviral driven-overexpression following a prolonged E2Fs1/3 DKO period could further show Ascl1 can rescue neurogenesis by bypassing the regulatory requirement of E2Fs1/3.

Additional effects of activator E2Fs1/3 on different NSC sub-types have yet to be fully characterized. Novel sequencing and bioinformatic techniques are used to further

sub-divide adult NSCs into respective clusters each with unique properties and purpose for neurogenesis (Llorens-Bobadilla et al., 2015; Shin et al., 2015). Based on the characterization results in this paper, it is possible that E2Fs1/3 affect NSCs at their most basic, primitive stage, and prime these cells towards either activation or be involved in determining a certain fate or both. Additionally, E2Fs1/3 may be the prime regulator of NSC activity being downstream in the Rb/E2F pathway. To elucidate this, additional bioinformatic analysis may be required to tie Rb/E2F pathway activity to specific sub-types of NSCs. Online repositories and published transcriptome analysis of various NSC sub-types could be overlaid with bioinformatic data derived from E2Fs1/3 DKO NSC RNA. Together, these kinds of correlative analyses could suggest specificity of E2Fs1/3 to certain NSC sub-types, and suggest whether E2F activity is required only for some NSC sub-types and not others. Keeping in mind that such analyses are purely correlative, additional experiments targeting molecular mechanisms would be required.

While this thesis has demonstrated a requirement for E2Fs1/3 in healthy adult neurogenesis, the requirement has not yet been shown in models of increased neurogenesis, such as either disease- or exercise-enhanced neurogenesis. It is well-established that neurogenesis and NSC activity increases following acute brain injury, such as stroke (Arvidsson et al., 2002; Dibajnia and Morshead, 2013; Faiz et al., 2015). The role of E2Fs1/3 in disease-enhanced neurogenesis is yet to be established. Inducing stroke in animals following double-knockout of E2Fs1/3, and observing whether NSCs can activate can yield further answers about whether E2Fs1/3 are unique regulators of NSC activation, or can be bypassed by other mechanisms triggered by different mechanisms. Additionally, experiments that seek to enhance the inherent neurogenic

response of the adult NSC niches can be repeated with running-wheel experiments, where E2Fs1/3 deficient mice are allowed free-access to cardiac exercise known to enhance neurogenesis (Blackmore et al., 2009). Such experiments serve to elucidate whether E2Fs1/3 are the primary regulators of NSC activation and exit from quiescence and thus cannot be bypassed by any mechanisms, or there exist other factors which can modulate neurogenic activity independent of the cell-cycle Rb/E2F pathway.

As E2Fs1/3 deficiency presents a clear deficit in healthy adult neurogenesis, it is pertinent to explore the behavioral impact of the phenotype. DKO mice put through various behavioral memory testing, such as the Morris Water Maze (MWM) or Fear Conditioning (FC) could reveal the behavioral consequences of ablating neurogenesis in the adult brain (Becker and Wojtowicz, 2007; Deng et al., 2010). In addition to pro-neural activation, results of such behavioral studies could possess significant consequences for the modulation of cognition and behavior, and further clinical study into the behavior of activator E2Fs1/3 in patients with under-activation of NSCs and impaired neurogenesis.

CONCLUSION

Our findings demonstrate that E2Fs1/3 are required for **adult neural stem cell exit from quiescence and entry into the cell-cycle**. Characterization of the neurogenic niches following a combined knockout of germline E2F1 and conditional E2F3 reveals a remarkable shutdown of neurogenesis and stem cell activation. Additionally, we have discovered that pro-neural gene Ascl1 is reduced following our knockout, leading us to propose that pro-neural activation is significantly affected by activator E2Fs1/3 activity. Overall, the lack of activation among neural stem cells and the ablation of progenitors and newborn neurons leads us to suggest that E2Fs1/3 plays a pivotal role in the regulation of neurogenesis and stem cell maintenance.

REFERENCES

Aguirre, A., Rubio, M.E., and Gallo, V. (2010). Notch and EGFR pathway interaction regulates neural stem cell number and self-renewal. Nature *467*, 323–327.

Aimone, J.B., Li, Y., Lee, S.W., Clemenson, G.D., Deng, W., and Gage, F.H. (2014). Regulation and Function of Adult Neurogenesis: From Genes to Cognition. Physiological Reviews *94*, 991–1026.

Akers, K.G., Martinez-Canabal, A., Restivo, L., Yiu, A.P., Cristofaro, A.D., Hsiang, H.-L. (Liz), Wheeler, A.L., Guskjolen, A., Niibori, Y., Shoji, H., et al. (2014). Hippocampal Neurogenesis Regulates Forgetting During Adulthood and Infancy. Science *344*, 598–602.

Aksoy, O., Chicas, A., Zeng, T., Zhao, Z., McCurrach, M., Wang, X., and Lowe, S.W. (2012). The atypical E2F family member E2F7 couples the p53 and RB pathways during cellular senescence. Genes Dev. *26*, 1546–1557.

Alexson, T.O., Hitoshi, S., Coles, B.L., Bernstein, A., and van der Kooy, D. (2006). Notch signaling is required to maintain all neural stem cell populations--irrespective of spatial or temporal niche. Dev. Neurosci. *28*, 34–48.

Altman, J., and Das, G.D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. Journal of Comparative Neurology *124*, 319–335.

Alvarez-Buylla, A., and García-Verdugo, J.M. (2002). Neurogenesis in Adult Subventricular Zone. J. Neurosci. *22*, 629–634.

Androutsellis-Theotokis, A., Leker, R.R., Soldner, F., Hoeppner, D.J., Ravin, R., Poser, S.W., Rueger, M.A., Bae, S.-K., Kittappa, R., and McKay, R.D.G. (2006). Notch signalling regulates stem cell numbers in vitro and in vivo. Nature *442*, 823–826.

Andrusiak, M.G., Vandenbosch, R., Park, D.S., and Slack, R.S. (2012). The Retinoblastoma Protein Is Essential for Survival of Postmitotic Neurons. J. Neurosci. *32*, 14809–14814.

Aranda-Anzaldo, A. (2012). The post-mitotic state in neurons correlates with a stable nuclear higher-order structure. Commun Integr Biol *5*, 134–139.

Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z., and Lindvall, O. (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. Nature Medicine *8*, 963–970.

Attwooll, C., Denchi, E.L., and Helin, K. (2004). The E2F family: specific functions and overlapping interests. EMBO J *23*, 4709–4716.

Becker, S., and Wojtowicz, J.M. (2007). A model of hippocampal neurogenesis in memory and mood disorders. Trends in Cognitive Sciences *11*, 70–76.

Bergmann, O., Liebl, J., Bernard, S., Alkass, K., Yeung, M.S.Y., Steier, P., Kutschera, W., Johnson, L., Landén, M., Druid, H., et al. (2012). The age of olfactory bulb neurons in humans. Neuron *74*, 634–639.

Bertoli, C., Skotheim, J.M., and de Bruin, R.A.M. (2013). Control of cell cycle transcription during G1 and S phases. Nat Rev Mol Cell Biol *14*, 518–528.

Beukelaers, P., Vandenbosch, R., Caron, N., Nguyen, L., Belachew, S., Moonen, G., Kiyokawa, H., Barbacid, M., Santamaria, D., and Malgrange, B. (2011). Cdk6-dependent regulation of G(1) length controls adult neurogenesis. Stem Cells *29*, 713–724.

Bjornson, C.R.R., Cheung, T.H., Liu, L., Tripathi, P.V., Steeper, K.M., and Rando, T.A. (2012). Notch signaling is necessary to maintain quiescence in adult muscle stem cells. Stem Cells *30*, 232–242.

Blackmore, D.G., Golmohammadi, M.G., Large, B., Waters, M.J., and Rietze, R.L. (2009). Exercise increases neural stem cell number in a growth hormone-dependent manner, augmenting the regenerative response in aged mice. Stem Cells *27*, 2044–2052.

Blagosklonny, M.V. (2011). Cell cycle arrest is not senescence. Aging (Albany NY) *3*, 94–101.

Boldrini, M., Fulmore, C.A., Tartt, A.N., Simeon, L.R., Pavlova, I., Poposka, V., Rosoklija, G.B., Stankov, A., Arango, V., Dwork, A.J., et al. (2018). Human Hippocampal Neurogenesis Persists throughout Aging. Cell Stem Cell *22*, 589-599.e5.

Bonaguidi, M.A., McGuire, T., Hu, M., Kan, L., Samanta, J., and Kessler, J.A. (2005). LIF and BMP signaling generate separate and discrete types of GFAP-expressing cells. Development *132*, 5503–5514.

Brazel, C.Y., Limke, T.L., Osborne, J.K., Miura, T., Cai, J., Pevny, L., and Rao, M.S. (2005). Sox2 expression defines a heterogeneous population of neurosphere-forming cells in the adult murine brain. Aging Cell *4*, 197–207.

Calegari, F., and Huttner, W.B. (2003). An inhibition of cyclin-dependent kinases that lengthens, but does not arrest, neuroepithelial cell cycle induces premature neurogenesis. J. Cell. Sci. *116*, 4947–4955.

Calegari, F., Haubensak, W., Haffner, C., and Huttner, W.B. (2005). Selective lengthening of the cell cycle in the neurogenic subpopulation of neural progenitor cells during mouse brain development. J. Neurosci. *25*, 6533–6538.

Cameron, H.A., and Glover, L.R. (2015). Adult Neurogenesis: Beyond Learning and Memory. Annu. Rev. Psychol. *66*, 53–81.

Casarosa, S., Fode, C., and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. Development *126*, 525–534.

Castro, D.S., Martynoga, B., Parras, C., Ramesh, V., Pacary, E., Johnston, C., Drechsel, D., Lebel-Potter, M., Garcia, L.G., Hunt, C., et al. (2011). A novel function of the proneural factor Ascl1 in progenitor proliferation identified by genome-wide characterization of its targets. Genes Dev *25*, 930–945.

Chagastelles, P.C., and Nardi, N.B. (2011). Biology of stem cells: an overview. Kidney Int Suppl (2011) *1*, 63–67.

Chavali, M., Klingener, M., Kokkosis, A.G., Garkun, Y., Felong, S., Maffei, A., and Aguirre, A. (2018). Non-canonical Wnt signaling regulates neural stem cell quiescence during homeostasis and after demyelination. Nat Commun *9*, 36.

Chazal, G., Durbec, P., Jankovski, A., Rougon, G., and Cremer, H. (2000). Consequences of neural cell adhesion molecule deficiency on cell migration in the rostral migratory stream of the mouse. J. Neurosci. *20*, 1446–1457.

Chen, D., Livne-bar, I., Vanderluit, J.L., Slack, R.S., Agochiya, M., and Bremner, R. (2004). Cell-specific effects of RB or RB/p107 loss on retinal development implicate an intrinsically death-resistant cell-of-origin in retinoblastoma. Cancer Cell *5*, 539–551.

Chen, D., Pacal, M., Wenzel, P., Knoepfler, P.S., Leone, G., and Bremner, R. (2009a). Division and apoptosis of E2f-deficient retinal progenitors. Nature *462*, 925–929.

Chen, H., Thiagalingam, A., Chopra, H., Borges, M.W., Feder, J.N., Nelkin, B.D., Baylin, S.B., and Ball, D.W. (1997). Conservation of the Drosophila lateral inhibition pathway in human lung cancer: a hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. Proc. Natl. Acad. Sci. U.S.A. *94*, 5355–5360.

Chen, H.-Z., Tsai, S.-Y., and Leone, G. (2009b). Emerging roles of E2Fs in cancer: an exit from cell cycle control. Nat Rev Cancer *9*, 785–797.

Choi, Y.J., and Anders, L. (2014). Signaling through cyclin D-dependent kinases. Oncogene *33*, 1890–1903.

Chong, J.-L., Wenzel, P.L., Sáenz-Robles, M.T., Nair, V., Ferrey, A., Hagan, J.P., Gomez, Y.M., Sharma, N., Chen, H.-Z., Ouseph, M., et al. (2009). E2f1-3 switch from activators in progenitor cells to repressors in differentiating cells. Nature *462*, 930–934.

Cicero, S.A., Johnson, D., Reyntjens, S., Frase, S., Connell, S., Chow, L.M.L., Baker, S.J., Sorrentino, B.P., and Dyer, M.A. (2009). Cells previously identified as retinal stem cells are pigmented ciliary epithelial cells. PNAS *106*, 6685–6690.

Claudio, P.P., Howard, C.M., Baldi, A., Luca, A.D., Fu, Y., Condorelli, G., Sun, Y., Colburn, N., Calabretta, B., and Giordano, A. (1994). p130/pRb2 Has Growth

Suppressive Properties Similar to yet Distinctive from Those of Retinoblastoma Family Members pRb and p107. Cancer Res *54*, 5556–5560.

Clemente Motta-Teixeira, L., Takada, S.H., Machado-Nils, A.V., Nogueira, M.I., and Xavier, G.F. (2015). Spatial learning and neurogenesis: Effects of cessation of wheel running and survival of novel neurons by engagement in cognitive tasks. Hippocampus.

Codega, P., Silva-Vargas, V., Paul, A., Maldonado-Soto, A.R., DeLeo, A.M., Pastrana, E., and Doetsch, F. (2014). Prospective Identification and Purification of Quiescent Adult Neural Stem Cells from Their In Vivo Niche. Neuron *82*, 545–559.

Cooper-Kuhn, C.M., Vroemen, M., Brown, J., Ye, H., Thompson, M.A., Winkler, J., and Kuhn, H.G. (2002). Impaired adult neurogenesis in mice lacking the transcription factor E2F1. Mol. Cell. Neurosci. *21*, 312–323.

Corbeil, H.B., Whyte, P., and Branton, P.E. (1995). Characterization of transcription factor E2F complexes during muscle and neuronal differentiation. Oncogene *11*, 909–920.

Curtis, M.A., Kam, M., Nannmark, U., Anderson, M.F., Axell, M.Z., Wikkelso, C., Holtås, S., Roon-Mom, W.M.C. van, Björk-Eriksson, T., Nordborg, C., et al. (2007). Human Neuroblasts Migrate to the Olfactory Bulb via a Lateral Ventricular Extension. Science *315*, 1243–1249.

Dannenberg, J.-H., Schuijff, L., Dekker, M., van der Valk, M., and Riele, H. te (2004). Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev *18*, 2952–2962.

Daynac, M., Tirou, L., Faure, H., Mouthon, M.-A., Gauthier, L.R., Hahn, H., Boussin, F.D., and Ruat, M. (2016). Hedgehog Controls Quiescence and Activation of Neural Stem Cells in the Adult Ventricular-Subventricular Zone. Stem Cell Reports *7*, 735–748.

DeGregori, J., and Johnson, D.G. (2006). Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. Curr. Mol. Med. *6*, 739–748.

Dehay, C., and Kennedy, H. (2007). Cell-cycle control and cortical development. Nat. Rev. Neurosci. *8*, 438–450.

Deng, W., Aimone, J.B., and Gage, F.H. (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat. Rev. Neurosci. *11*, 339–350.

Dibajnia, P., and Morshead, C.M. (2013). Role of neural precursor cells in promoting repair following stroke. Acta Pharmacol Sin *34*, 78–90.

Dimri, G.P., Itahana, K., Acosta, M., and Campisi, J. (2000). Regulation of a senescence checkpoint response by the E2F1 transcription factor and p14(ARF) tumor suppressor. Mol. Cell. Biol. *20*, 273–285.

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. Genes Dev. *12*, 2245–2262.

Emsley, J.G., and Hagg, T. (2003). alpha6beta1 integrin directs migration of neuronal precursors in adult mouse forebrain. Exp. Neurol. *183*, 273–285.

Encinas, J.M., Michurina, T.V., Peunova, N., Park, J.-H., Tordo, J., Peterson, D.A., Fishell, G., Koulakov, A., and Enikolopov, G. (2011). Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. Cell Stem Cell *8*, 566–579.

Engler, A., Rolando, C., Giachino, C., Saotome, I., Erni, A., Brien, C., Zhang, R., Zimber-Strobl, U., Radtke, F., Artavanis-Tsakonas, S., et al. (2018). Notch2 Signaling Maintains NSC Quiescence in the Murine Ventricular-Subventricular Zone. Cell Rep *22*, 992–1002.

Fabel, K., Wolf, S.A., Ehninger, D., Babu, H., Leal-Galicia, P., and Kempermann, G. (2009). Additive Effects of Physical Exercise and Environmental Enrichment on Adult Hippocampal Neurogenesis in Mice. Front Neurosci *3*.

Faiz, M., Sachewsky, N., Gascón, S., Bang, K.W.A., Morshead, C.M., and Nagy, A. (2015). Adult Neural Stem Cells from the Subventricular Zone Give Rise to Reactive Astrocytes in the Cortex after Stroke. Cell Stem Cell *17*, 624–634.

Fajas, L., Landsberg, R.L., Huss-Garcia, Y., Sardet, C., Lees, J.A., and Auwerx, J. (2002). E2Fs regulate adipocyte differentiation. Dev. Cell *3*, 39–49.

Farah, M.H., Olson, J.M., Sucic, H.B., Hume, R.I., Tapscott, S.J., and Turner, D.L. (2000). Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. Development *127*, 693–702.

Field, S.J., Tsai, F.Y., Kuo, F., Zubiaga, A.M., Kaelin, W.G., Livingston, D.M., Orkin, S.H., and Greenberg, M.E. (1996). E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell *85*, 549–561.

Fischer, M., and Müller, G.A. (2017). Cell cycle transcription control: DREAM/MuvB and RB-E2F complexes. Critical Reviews in Biochemistry and Molecular Biology *52*, 638–662.

Frade, J.M., and Ovejero-Benito, M.C. (2015). Neuronal cell cycle: the neuron itself and its circumstances. Cell Cycle *14*, 712–720.

Frielingsdorf, H., and Kuhn, H.G. (2007). Adult neurogenesis—a reality check. Debates in Neuroscience *1*, 33–41.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M., and Dryja, T.P. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature *323*, 643–646.

Fuentealba, L.C., Rompani, S.B., Parraguez, J.I., Obernier, K., Romero, R., Cepko, C.L., and Alvarez-Buylla, A. (2015). Embryonic Origin of Postnatal Neural Stem Cells. Cell *161*, 1644–1655.

Furutachi, S., Miya, H., Watanabe, T., Kawai, H., Yamasaki, N., Harada, Y., Imayoshi, I., Nelson, M., Nakayama, K.I., Hirabayashi, Y., et al. (2015). Slowly dividing neural progenitors are an embryonic origin of adult neural stem cells. Nat Neurosci *18*, 657–665.

Garthe, A., and Kempermann, G. (2013). An old test for new neurons: refining the Morris water maze to study the functional relevance of adult hippocampal neurogenesis. Front. Neurosci. *7*.

Ge, S., Yang, C.-H., Hsu, K.-S., Ming, G.-L., and Song, H. (2007). A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. Neuron *54*, 559–566.

Genander, M., and Frisén, J. (2010). Ephrins and Eph receptors in stem cells and cancer. Curr. Opin. Cell Biol. *22*, 611–616.

Gheusi, G., Cremer, H., McLean, H., Chazal, G., Vincent, J.D., and Lledo, P.M. (2000). Importance of newly generated neurons in the adult olfactory bulb for odor discrimination. Proc. Natl. Acad. Sci. U.S.A. *97*, 1823–1828.

Giacinti, C., and Giordano, A. (2006). RB and cell cycle progression. Oncogene *25*, 5220–5227.

Groves, J.O., Leslie, I., Huang, G.-J., McHugh, S.B., Taylor, A., Mott, R., Munafò, M., Bannerman, D.M., and Flint, J. (2013). Ablating Adult Neurogenesis in the Rat Has No Effect on Spatial Processing: Evidence from a Novel Pharmacogenetic Model. PLOS Genetics *9*, e1003718.

Guillemot, F., and Joyner, A.L. (1993). Dynamic expression of the murine Achaete-Scute homologue Mash-1 in the developing nervous system. Mech. Dev. *42*, 171–185.

Han, Y.-G., Spassky, N., Romaguera-Ros, M., Garcia-Verdugo, J.-M., Aguilar, A., Schneider-Maunoury, S., and Alvarez-Buylla, A. (2008). Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells. Nat. Neurosci. *11*, 277–284.

Harbour, J.W. (1998). Overview of RB gene mutations in patients with retinoblastoma - Implications for clinical genetic screening. Ophthalmology *105*, 1442–1447.

Harbour, J.W., and Dean, D.C. (2000). The Rb/E2F pathway: expanding roles and emerging paradigms. Genes Dev. *14*, 2393–2409.

Harbour, J.W., Luo, R.X., Santi, A.D., Postigo, A.A., and Dean, D.C. (1999). Cdk Phosphorylation Triggers Sequential Intramolecular Interactions that Progressively Block Rb Functions as Cells Move through G1. Cell *98*, 859–869.

Helin, K., Holm, K., Niebuhr, A., Eiberg, H., Tommerup, N., Hougaard, S., Poulsen, H.S., Spang-Thomsen, M., and Nørgaard, P. (1997). Loss of the retinoblastoma protein-related p130 protein in small cell lung carcinoma. PNAS *94*, 6933–6938.

Hirota, Y., Ohshima, T., Kaneko, N., Ikeda, M., Iwasato, T., Kulkarni, A.B., Mikoshiba, K., Okano, H., and Sawamoto, K. (2007). Cyclin-Dependent Kinase 5 Is Required for Control of Neuroblast Migration in the Postnatal Subventricular Zone. J. Neurosci. *27*, 12829–12838.

Hitoshi, S., Alexson, T., Tropepe, V., Donoviel, D., Elia, A.J., Nye, J.S., Conlon, R.A., Mak, T.W., Bernstein, A., and van der Kooy, D. (2002). Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. Genes Dev *16*, 846–858.

Hodge, R.D., D'Ercole, A.J., and O'Kusky, J.R. (2004). Insulin-like growth factor-I accelerates the cell cycle by decreasing G1 phase length and increases cell cycle reentry in the embryonic cerebral cortex. J. Neurosci. *24*, 10201–10210.

Hodge, R.D., Kowalczyk, T.D., Wolf, S.A., Encinas, J.M., Rippey, C., Enikolopov, G., Kempermann, G., and Hevner, R.F. (2008). Intermediate progenitors in adult hippocampal neurogenesis: Tbr2 expression and coordinate regulation of neuronal output. J. Neurosci. *28*, 3707–3717.

Hodge, R.D., Nelson, B.R., Kahoud, R.J., Yang, R., Mussar, K.E., Reiner, S.L., and Hevner, R.F. (2012). Tbr2 is essential for hippocampal lineage progression from neural stem cells to intermediate progenitors and neurons. J Neurosci *32*, 6275–6287.

Höglinger, G.U., Breunig, J.J., Depboylu, C., Rouaux, C., Michel, P.P., Alvarez-Fischer, D., Boutillier, A.-L., Degregori, J., Oertel, W.H., Rakic, P., et al. (2007). The pRb/E2F cell-cycle pathway mediates cell death in Parkinson's disease. Proc. Natl. Acad. Sci. U.S.A. *104*, 3585–3590.

Horowitz, J.M., Park, S.H., Bogenmann, E., Cheng, J.C., Yandell, D.W., Kaye, F.J., Minna, J.D., Dryja, T.P., and Weinberg, R.A. (1990). Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. PNAS *87*, 2775–2779.

Hsieh, J.K., Fredersdorf, S., Kouzarides, T., Martin, K., and Lu, X. (1997). E2F1-induced apoptosis requires DNA binding but not transactivation and is inhibited by the retinoblastoma protein through direct interaction. Genes Dev. *11*, 1840–1852.

Huang, H.J., Yee, J.K., Shew, J.Y., Chen, P.L., Bookstein, R., Friedmann, T., Lee, E.Y., and Lee, W.H. (1988). Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. Science *242*, 1563–1566.

Humbert, P.O., Verona, R., Trimarchi, J.M., Rogers, C., Dandapani, S., and Lees, J.A. (2000). E2f3 is critical for normal cellular proliferation. Genes Dev. *14*, 690–703.

Hurford, R.K., Cobrinik, D., Lee, M.H., and Dyson, N. (1997). pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. Genes Dev. *11*, 1447–1463.

Iaquinta, P.J., and Lees, J.A. (2007). Life and death decisions by the E2F transcription factors. Curr Opin Cell Biol *19*, 649–657.

Ikeda, M.A., Jakoi, L., and Nevins, J.R. (1996). A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. Proc. Natl. Acad. Sci. U.S.A. *93*, 3215–3220.

Imayoshi, I., Sakamoto, M., Yamaguchi, M., Mori, K., and Kageyama, R. (2010). Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. J. Neurosci. *30*, 3489–3498.

Ishibashi, M., Ang, S.L., Shiota, K., Nakanishi, S., Kageyama, R., and Guillemot, F. (1995). Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev. *9*, 3136–3148.

Jablonska, B., Aguirre, A., Vandenbosch, R., Belachew, S., Berthet, C., Kaldis, P., and Gallo, V. (2007). Cdk2 is critical for proliferation and self-renewal of neural progenitor cells in the adult subventricular zone. J. Cell Biol. *179*, 1231–1245.

Jessberger, S., Aigner, S., Clemenson, G.D., Toni, N., Lie, D.C., Karalay, O., Overall, R., Kempermann, G., and Gage, F.H. (2008). Cdk5 regulates accurate maturation of newborn granule cells in the adult hippocampus. PLoS Biol. *6*, e272.

Julian, L.M., and Blais, A. (2015). Transcriptional control of stem cell fate by E2Fs and pocket proteins. Front Genet *6*.

Julian, L.M., Vandenbosch, R., Pakenham, C.A., Andrusiak, M.G., Nguyen, A.P., McClellan, K.A., Svoboda, D.S., Lagace, D.C., Park, D.S., Leone, G., et al. (2013). Opposing regulation of Sox2 by cell-cycle effectors E2f3a and E2f3b in neural stem cells. Cell Stem Cell *12*, 440–452.

Kamijo, T., Zindy, F., Roussel, M.F., Quelle, D.E., Downing, J.R., Ashmun, R.A., Grosveld, G., and Sherr, C.J. (1997). Tumor Suppression at the Mouse INK4a Locus Mediated by the Alternative Reading Frame Product p19 ARF. Cell *91*, 649–659.

Kele, J., Simplicio, N., Ferri, A.L.M., Mira, H., Guillemot, F., Arenas, E., and Ang, S.-L. (2006). Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. Development *133*, 495–505.

Kempermann, G., Kuhn, H.G., and Gage, F.H. (1997). More hippocampal neurons in adult mice living in an enriched environment. Nature *386*, 493–495.

Kempermann, G., Gage, F.H., Aigner, L., Song, H., Curtis, M.A., Thuret, S., Kuhn, H.G., Jessberger, S., Frankland, P.W., Cameron, H.A., et al. (2018). Human Adult Neurogenesis: Evidence and Remaining Questions. Cell Stem Cell *23*, 25–30.

Khacho, M., Clark, A., Svoboda, D.S., Azzi, J., MacLaurin, J.G., Meghaizel, C., Sesaki, H., Lagace, D.C., Germain, M., Harper, M.-E., et al. (2016). Mitochondrial Dynamics Impacts Stem Cell Identity and Fate Decisions by Regulating a Nuclear Transcriptional Program. Cell Stem Cell *19*, 232–247.

Kim, E.J., Ables, J.L., Dickel, L.K., Eisch, A.J., and Johnson, J.E. (2011). Ascl1 (Mash1) Defines Cells with Long-Term Neurogenic Potential in Subgranular and Subventricular Zones in Adult Mouse Brain. PLoS One *6*.

King, J.C., Moskowitz, I.P.G., Burgon, P.G., Ahmad, F., Stone, J.R., Seidman, J.G., and Lees, J.A. (2008). E2F3 plays an essential role in cardiac development and function. Cell Cycle *7*, 3775–3780.

Kong, L.-J., Chang, J.T., Bild, A.H., and Nevins, J.R. (2007). Compensation and specificity of function within the E2F family. Oncogene *26*, 321–327.

Kowalczyk, A., Filipkowski, R.K., Rylski, M., Wilczynski, G.M., Konopacki, F.A., Jaworski, J., Ciemerych, M.A., Sicinski, P., and Kaczmarek, L. (2004). The critical role of cyclin D2 in adult neurogenesis. J. Cell Biol. *167*, 209–213.

Kowalik, T.F., DeGregori, J., Schwarz, J.K., and Nevins, J.R. (1995). E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. J. Virol. *69*, 2491–2500.

Kriegstein, A., and Alvarez-Buylla, A. (2009). The Glial Nature of Embryonic and Adult Neural Stem Cells. Annu Rev Neurosci *32*, 149–184.

Krimpenfort, P., Quon, K.C., Mooi, W.J., Loonstra, A., and Berns, A. (2001). Loss of *p16^{Ink4a}* confers susceptibility to metastatic melanoma in mice. Nature *413*, 83–86.

Lacy, S., and Whyte, P. (1997). Identification of a p130 domain mediating interactions with cyclin A/cdk 2 and cyclin E/cdk 2 complexes. Oncogene *14*, 2395–2406.

Lagace, D.C., Whitman, M.C., Noonan, M.A., Ables, J.L., DeCarolis, N.A., Arguello, A.A., Donovan, M.H., Fischer, S.J., Farnbauch, L.A., Beech, R.D., et al. (2007). Dynamic contribution of nestin-expressing stem cells to adult neurogenesis. J. Neurosci. *27*, 12623–12629.

Lagace, D.C., Donovan, M.H., DeCarolis, N.A., Farnbauch, L.A., Malhotra, S., Berton, O., Nestler, E.J., Krishnan, V., and Eisch, A.J. (2010). Adult hippocampal neurogenesis is functionally important for stress-induced social avoidance. PNAS 200910072.

Lange, C., and Calegari, F. (2010). Cdks and cyclins link G1 length and differentiation of embryonic, neural and hematopoietic stem cells. Cell Cycle *9*, 1893–1900.

Lange, C., Huttner, W.B., and Calegari, F. (2009). Cdk4/cyclinD1 overexpression in neural stem cells shortens G1, delays neurogenesis, and promotes the generation and expansion of basal progenitors. Cell Stem Cell *5*, 320–331.

Lazzerini Denchi, E., and Helin, K. (2005). E2F1 is crucial for E2F-dependent apoptosis. EMBO Rep *6*, 661–667.

Le Dréau, G., Escalona, R., Fueyo, R., Herrera, A., Martínez, J.D., Usieto, S., Menendez, A., Pons, S., Martinez-Balbas, M.A., and Marti, E. (2018). E proteins sharpen neurogenesis by modulating proneural bHLH transcription factors' activity in an E-box-dependent manner. ELife *7*, e37267.

Lee, J.-K., Cho, J.-H., Hwang, W.-S., Lee, Y.-D., Reu, D.-S., and Suh-Kim, H. (2000). Expression of neuroD/BETA2 in mitotic and postmitotic neuronal cells during the development of nervous system. Developmental Dynamics *217*, 361–367.

Lee, W.H., Bookstein, R., Hong, F., Young, L.J., Shew, J.Y., and Lee, E.Y. (1987). Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science *235*, 1394–1399.

Leemans, C.R., Braakhuis, B.J.M., and Brakenhoff, R.H. (2011). The molecular biology of head and neck cancer. Nat Rev Cancer *11*, 9–22.

Lehmann, M.L., Brachman, R.A., Martinowich, K., Schloesser, R.J., and Herkenham, M. (2013). Glucocorticoids orchestrate divergent effects on mood through adult neurogenesis. J Neurosci *33*, 2961–2972.

Leng, X., Connell-Crowley, L., Goodrich, D., and Harper, J.W. (1997). S-Phase entry upon ectopic expression of G1 cyclin-dependent kinases in the absence of retinoblastoma protein phosphorylation. Current Biology *7*, 709–712.

Levine, A.J. (1997). p53, the Cellular Gatekeeper for Growth and Division. Cell *88*, 323–331.

Li, G., Fang, L., Fernández, G., and Pleasure, S.J. (2013). The ventral hippocampus is the embryonic origin for adult neural stem cells in the dentate gyrus. Neuron *78*, 658–672.

Lie, D.-C., Colamarino, S.A., Song, H.-J., Désiré, L., Mira, H., Consiglio, A., Lein, E.S., Jessberger, S., Lansford, H., Dearie, A.R., et al. (2005). Wnt signalling regulates adult hippocampal neurogenesis. Nature *437*, 1370–1375.

Lim, S., and Kaldis, P. (2012). Loss of Cdk2 and Cdk4 induces a switch from proliferation to differentiation in neural stem cells. Stem Cells *30*, 1509–1520.

Lim, S., Bhinge, A., Alonso, S.B., Aksoy, I., Aprea, J., Cheok, C.F., Calegari, F., Stanton, L.W., and Kaldis, P. (2017). Cyclin-Dependent Kinase-Dependent Phosphorylation of Sox2 at Serine 39 Regulates Neurogenesis. Molecular and Cellular Biology *37*, e00201-17.

Llorens-Bobadilla, E., Zhao, S., Baser, A., Saiz-Castro, G., Zwadlo, K., and Martin-Villalba, A. (2015). Single-Cell Transcriptomics Reveals a Population of Dormant Neural Stem Cells that Become Activated upon Brain Injury. Cell Stem Cell *17*, 329–340.

Lo, L.C., Johnson, J.E., Wuenschell, C.W., Saito, T., and Anderson, D.J. (1991). Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. Genes Dev. *5*, 1524–1537.

Lois, C., and Alvarez-Buylla, A. (1994). Long-distance neuronal migration in the adult mammalian brain. Science *264*, 1145–1148.

Lugert, S., Basak, O., Knuckles, P., Haussler, U., Fabel, K., Götz, M., Haas, C.A., Kempermann, G., Taylor, V., and Giachino, C. (2010). Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. Cell Stem Cell *6*, 445–456.

Lukas, J., Herzinger, T., Hansen, K., Moroni, M.C., Resnitzky, D., Helin, K., Reed, S.I., and Bartek, J. (1997). Cyclin E-induced S phase without activation of the pRb/E2F pathway. Genes Dev. *11*, 1479–1492.

Lukaszewicz, A., Savatier, P., Cortay, V., Kennedy, H., and Dehay, C. (2002). Contrasting effects of basic fibroblast growth factor and neurotrophin 3 on cell cycle kinetics of mouse cortical stem cells. J. Neurosci. *22*, 6610–6622.

MacPherson, D., Sage, J., Kim, T., Ho, D., McLaughlin, M.E., and Jacks, T. (2004). Cell type-specific effects of Rb deletion in the murine retina. Genes Dev. *18*, 1681–1694.

MacQueen, G.M., Campbell, S., McEwen, B.S., Macdonald, K., Amano, S., Joffe, R.T., Nahmias, C., and Young, L.T. (2003). Course of illness, hippocampal function, and hippocampal volume in major depression. Proc Natl Acad Sci U S A *100*, 1387–1392.

Mak, G.K., and Weiss, S. (2010). Paternal recognition of adult offspring mediated by newly generated CNS neurons. Nature Neuroscience *13*, 753–758.

Martinez, L.A., Goluszko, E., Chen, H.-Z., Leone, G., Post, S., Lozano, G., Chen, Z., and Chauchereau, A. (2010). E2F3 Is a Mediator of DNA Damage-Induced Apoptosis. Molecular and Cellular Biology *30*, 524–536.

McClellan, K.A., and Slack, R.S. (2007). Specific In Vivo Roles for E2Fs in Differentiation and Development. Cell Cycle *6*, 2917–2927.

McClellan, K.A., Ruzhynsky, V.A., Douda, D.N., Vanderluit, J.L., Ferguson, K.L., Chen, D., Bremner, R., Park, D.S., Leone, G., and Slack, R.S. (2007). Unique Requirement for Rb/E2F3 in Neuronal Migration: Evidence for Cell Cycle-Independent Functions. Molecular and Cellular Biology *27*, 4825–4843.

Mich, J.K., Signer, R.A., Nakada, D., Pineda, A., Burgess, R.J., Vue, T.Y., Johnson, J.E., and Morrison, S.J. (2014). Prospective identification of functionally distinct stem cells and neurosphere-initiating cells in adult mouse forebrain. ELife *3*.

Ming, G.-L., and Song, H. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron *70*, 687–702.

Mizutani, K., Yoon, K., Dang, L., Tokunaga, A., and Gaiano, N. (2007). Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. Nature *449*, 351–355.

Moberg, K., Starz, M.A., and Lees, J.A. (1996). E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. Mol Cell Biol *16*, 1436–1449.

Morizur, L., Chicheportiche, A., Gauthier, L.R., Daynac, M., Boussin, F.D., and Mouthon, M.-A. (2018). Distinct Molecular Signatures of Quiescent and Activated Adult Neural Stem Cells Reveal Specific Interactions with Their Microenvironment. Stem Cell Reports *11*, 565–577.

Moroni, M.C., Hickman, E.S., Lazzerini Denchi, E., Caprara, G., Colli, E., Cecconi, F., Müller, H., and Helin, K. (2001). Apaf-1 is a transcriptional target for E2F and p53. Nat. Cell Biol. *3*, 552–558.

Mu, L., Berti, L., Masserdotti, G., Covic, M., Michaelidis, T.M., Doberauer, K., Merz, K., Rehfeld, F., Haslinger, A., Wegner, M., et al. (2012). SoxC Transcription Factors Are Required for Neuronal Differentiation in Adult Hippocampal Neurogenesis. J Neurosci *32*, 3067–3080.

Müller, H., Bracken, A.P., Vernell, R., Moroni, M.C., Christians, F., Grassilli, E., Prosperini, E., Vigo, E., Oliner, J.D., and Helin, K. (2001). E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev. *15*, 267–285.

Narasimha, A.M., Kaulich, M., Shapiro, G.S., Choi, Y.J., Sicinski, P., and Dowdy, S.F. (2014). Cyclin D activates the Rb tumor suppressor by mono-phosphorylation. ELife *3*.

Nevins, J.R. (2001). The Rb/E2F pathway and cancer. Hum Mol Genet *10*, 699–703.

Nguyen, L., Besson, A., Heng, J.I.-T., Schuurmans, C., Teboul, L., Parras, C., Philpott, A., Roberts, J.M., and Guillemot, F. (2006). p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. Genes Dev. *20*, 1511–1524.

Nguyen-Ba-Charvet, K.T., Picard-Riera, N., Tessier-Lavigne, M., Baron-Van Evercooren, A., Sotelo, C., and Chédotal, A. (2004). Multiple roles for slits in the control of cell migration in the rostral migratory stream. J. Neurosci. *24*, 1497–1506.

Nieto, M., Schuurmans, C., Britz, O., and Guillemot, F. (2001). Neural bHLH Genes Control the Neuronal versus Glial Fate Decision in Cortical Progenitors. Neuron *29*, 401–413.

Ouyang, J., Yu, W., Liu, J., Zhang, N., Florens, L., Chen, J., Liu, H., Washburn, M., Pei, D., and Xie, T. (2015). Cyclin-dependent kinase-mediated Sox2 phosphorylation enhances the ability of Sox2 to establish the pluripotent state. J. Biol. Chem. *290*, 22782–22794.

Paratcha, G., Ibáñez, C.F., and Ledda, F. (2006). GDNF is a chemoattractant factor for neuronal precursor cells in the rostral migratory stream. Mol. Cell. Neurosci. *31*, 505–514.

Parisi, T., Yuan, T.L., Faust, A.M., Caron, A.M., Bronson, R., and Lees, J.A. (2007). Selective Requirements for E2f3 in the Development and Tumorigenicity of Rb-Deficient Chimeric Tissues. Mol Cell Biol *27*, 2283–2293.

Paton, J.A., and Nottebohm, F.N. (1984). Neurons generated in the adult brain are recruited into functional circuits. Science *225*, 1046–1048.

Petreanu, L., and Alvarez-Buylla, A. (2002). Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. J. Neurosci. *22*, 6106–6113.

Pfister, J.A., Ma, C., Morrison, B.E., and D'Mello, S.R. (2008). Opposing Effects of Sirtuins on Neuronal Survival: SIRT1-Mediated Neuroprotection Is Independent of Its Deacetylase Activity. PLoS One *3*.

Phillips, A.C., Bates, S., Ryan, K.M., Helin, K., and Vousden, K.H. (1997). Induction of DNA synthesis and apoptosis are separable functions of E2F-1. Genes Dev. *11*, 1853–1863.

van Praag, H., Kempermann, G., and Gage, F.H. (1999). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci *2*, 266–270.

van Praag, H., Schinder, A.F., Christie, B.R., Toni, N., Palmer, T.D., and Gage, F.H. (2002). Functional neurogenesis in the adult hippocampus. Nature *415*, 1030–1034.

van Praag, H., Shubert, T., Zhao, C., and Gage, F.H. (2005). Exercise enhances learning and hippocampal neurogenesis in aged mice. J. Neurosci. *25*, 8680–8685.

Qin, X.Q., Livingston, D.M., Kaelin, W.G., and Adams, P.D. (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc. Natl. Acad. Sci. U.S.A. *91*, 10918–10922.

Qin, X.Q., Livingston, D.M., Ewen, M., Sellers, W.R., Arany, Z., and Kaelin, W.G. (1995). The transcription factor E2F-1 is a downstream target of RB action. Molecular and Cellular Biology *15*, 742–755.

Ramirez-Parra, E., López-Matas, M.A., Fründt, C., and Gutierrez, C. (2004). Role of an Atypical E2F Transcription Factor in the Control of Arabidopsis Cell Growth and Differentiation. The Plant Cell *16*, 2350–2363.

Reynolds, B.A., and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science *255*, 1707–1710.

Richards, L.J., Kilpatrick, T.J., and Bartlett, P.F. (1992). De novo generation of neuronal cells from the adult mouse brain. Proc. Natl. Acad. Sci. U.S.A. *89*, 8591–8595.

Rochefort, C., Gheusi, G., Vincent, J.-D., and Lledo, P.-M. (2002). Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. J. Neurosci. *22*, 2679–2689.

Ross, S.E., Greenberg, M.E., and Stiles, C.D. (2003). Basic Helix-Loop-Helix Factors in Cortical Development. Neuron *39*, 13–25.

Rotgers, E., Rivero-Müller, A., Nurmio, M., Parvinen, M., Guillou, F., Huhtaniemi, I., Kotaja, N., Bourguiba-Hachemi, S., and Toppari, J. (2014). Retinoblastoma protein (RB) interacts with E2F3 to control terminal differentiation of Sertoli cells. Cell Death Dis *5*, e1274.

Sage, J. (2012). The retinoblastoma tumor suppressor and stem cell biology. Genes Dev *26*, 1409–1420.

Salomoni, P., and Calegari, F. (2010). Cell cycle control of mammalian neural stem cells: putting a speed limit on G1. Trends Cell Biol. *20*, 233–243.

Sangwan, M., McCurdy, S.R., Livne-Bar, I., Ahmad, M., Wrana, J.L., Chen, D., and Bremner, R. (2012). Established and new mouse models reveal E2f1 and Cdk2 dependency of retinoblastoma, and expose effective strategies to block tumor initiation. Oncogene *31*, 5019–5028.

Sardet, C., LeCam, L., Fabbrizio, E., and Vidal, M. (1997). E2Fs and the Retinoblastoma Protein Family. In Oncogenes as Transcriptional Regulators: Cell Cycle Regulators and Chromosomal Translocation, M. Yaniv, and J. Ghysdael, eds. (Basel: Birkhäuser Basel), pp. 1–62.

Schafer, K.A. (1998). The Cell Cycle: A Review. Vet Pathol *35*, 461–478.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nature Methods *9*, 676–682.

Seib, D.R.M., Corsini, N.S., Ellwanger, K., Plaas, C., Mateos, A., Pitzer, C., Niehrs, C., Celikel, T., and Martin-Villalba, A. (2013). Loss of Dickkopf-1 restores neurogenesis in old age and counteracts cognitive decline. Cell Stem Cell *12*, 204–214.

Serrano, M., Lee, H.-W., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R.A. (1996). Role of the INK4a Locus in Tumor Suppression and Cell Mortality. Cell *85*, 27–37.

Sheline, Y.I., Sanghavi, M., Mintun, M.A., and Gado, M.H. (1999). Depression Duration But Not Age Predicts Hippocampal Volume Loss in Medically Healthy Women with Recurrent Major Depression. J. Neurosci. *19*, 5034–5043.

Shen, Q., Goderie, S.K., Jin, L., Karanth, N., Sun, Y., Abramova, N., Vincent, P., Pumiglia, K., and Temple, S. (2004). Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science *304*, 1338–1340.

Sherr, C.J. (1996). Cancer Cell Cycles. Science *274*, 1672–1677.

Sherr, C.J., and McCormick, F. (2002). The RB and p53 pathways in cancer. Cancer Cell *2*, 103–112.

Sherr, C.J., and Roberts, J.M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. *13*, 1501–1512.

Shin, J., Berg, D.A., Zhu, Y., Shin, J.Y., Song, J., Bonaguidi, M.A., Enikolopov, G., Nauen, D.W., Christian, K.M., Ming, G., et al. (2015). Single-Cell RNA-Seq with Waterfall Reveals Molecular Cascades underlying Adult Neurogenesis. Cell Stem Cell *17*, 360–372.

Soos, T.J., Kiyokawa, H., Yan, J.S., Rubin, M.S., Giordano, A., DeBlasio, A., Bottega, S., Wong, B., Mendelsohn, J., and Koff, A. (1996). Formation of p27-CDK complexes during the human mitotic cell cycle. Cell Growth Differ *7*, 135–146.

Sorrells, S.F., Paredes, M.F., Cebrian-Silla, A., Sandoval, K., Qi, D., Kelley, K.W., James, D., Mayer, S., Chang, J., Auguste, K.I., et al. (2018). Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. Nature *555*, 377–381.

Spalding, K.L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H.B., Boström, E., Westerlund, I., Vial, C., Buchholz, B.A., et al. (2013). Dynamics of Hippocampal Neurogenesis in Adult Humans. Cell *153*, 1219–1227.

Starostik, P., Chow, K.N., and Dean, D.C. (1996). Transcriptional repression and growth suppression by the p107 pocket protein. Molecular and Cellular Biology *16*, 3606–3614.

Sueda, R., Imayoshi, I., Harima, Y., and Kageyama, R. (2019). High Hes1 expression and resultant Ascl1 suppression regulate quiescent vs. active neural stem cells in the adult mouse brain. Genes Dev. *33*, 511–523.

Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T., and Dyke, T.V. (1994). p53-Dependent apoptosis suppresses tumor growth and progression in vivo. Cell *78*, 703–711.

Tavazoie, M., Van der Veken, L., Silva-Vargas, V., Louissaint, M., Colonna, L., Zaidi, B., Garcia-Verdugo, J.M., and Doetsch, F. (2008). A specialized vascular niche for adult neural stem cells. Cell Stem Cell *3*, 279–288.

Toda, T., and Gage, F.H. (2018). Review: adult neurogenesis contributes to hippocampal plasticity. Cell Tissue Res *373*, 693–709.

Toni, N., Teng, E.M., Bushong, E.A., Aimone, J.B., Zhao, C., Consiglio, A., van Praag, H., Martone, M.E., Ellisman, M.H., and Gage, F.H. (2007). Synapse formation on neurons born in the adult hippocampus. Nature Neuroscience *10*, 727–734.

Tozuka, Y., Fukuda, S., Namba, T., Seki, T., and Hisatsune, T. (2005). GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron *47*, 803–815.

Trimarchi, J.M., and Lees, J.A. (2002). Transcription: Sibling rivalry in the E2F family. Nature Reviews Molecular Cell Biology *3*, 11–20.

Tsai, K.Y., Hu, Y., Macleod, K.F., Crowley, D., Yamasaki, L., and Jacks, T. (1998). Mutation of E2f-1 Suppresses Apoptosis and Inappropriate S Phase Entry and Extends Survival of Rb-Deficient Mouse Embryos. Molecular Cell *2*, 293–304.

Urbán, N., and Guillemot, F. (2014). Neurogenesis in the embryonic and adult brain: same regulators, different roles. Front Cell Neurosci *8*.

Urbán, N., van den Berg, D.L.C., Forget, A., Andersen, J., Demmers, J.A.A., Hunt, C., Ayrault, O., and Guillemot, F. (2016). Return to quiescence of mouse neural stem cells by degradation of a proactivation protein. Science *353*, 292–295.

Vandenbosch, R., Clark, A., Fong, B.C., Omais, S., Jaafar, C., Dugal-Tessier, D., Dhaliwal, J., Lagace, D.C., Park, D.S., Ghanem, N., et al. (2016). RB regulates the production and the survival of newborn neurons in the embryonic and adult dentate gyrus. Hippocampus *26*, 1379–1392.

Vanderluit, J.L., Wylie, C.A., McClellan, K.A., Ghanem, N., Fortin, A., Callaghan, S., MacLaurin, J.G., Park, D.S., and Slack, R.S. (2007). The Retinoblastoma family member p107 regulates the rate of progenitor commitment to a neuronal fate. J Cell Biol *178*, 129–139.

Vasavada, R.C., Cozar-Castellano, I., Sipula, D., and Stewart, A.F. (2007). Tissue-Specific Deletion of the Retinoblastoma Protein in the Pancreatic β-Cell Has Limited Effects on β-Cell Replication, Mass, and Function. Diabetes *56*, 57–64.

Vasconcelos, F.F., and Castro, D.S. (2014). Transcriptional control of vertebrate neurogenesis by the proneural factor Ascl1. Front Cell Neurosci *8*.

Vernon, A.E., Devine, C., and Philpott, A. (2003). The cdk inhibitor p27Xic1 is required for differentiation of primary neurones in Xenopus. Development *130*, 85–92.

Viatour, P., Somervaille, T.C., Venkatasubrahmanyam, S., Kogan, S., McLaughlin, M.E., Weissman, I.L., Butte, A.J., Passegué, E., and Sage, J. (2008). Hematopoietic stem cell quiescence is maintained by compound contributions of the retinoblastoma gene family. Cell Stem Cell *3*, 416–428.

Wang, C., Chen, L., Hou, X., Li, Z., Kabra, N., Ma, Y., Nemoto, S., Finkel, T., Gu, W., Cress, W.D., et al. (2006). Interactions between E2F1 and SirT1 regulate apoptotic response to DNA damage. Nat. Cell Biol. *8*, 1025–1031.

Ward, N.L., and Lamanna, J.C. (2004). The neurovascular unit and its growth factors: coordinated response in the vascular and nervous systems. Neurol. Res. *26*, 870–883.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323–330.

Wu, C.L., Classon, M., Dyson, N., and Harlow, E. (1996). Expression of dominant-negative mutant DP-1 blocks cell cycle progression in G1. Molecular and Cellular Biology *16*, 3698–3706.

Wu, L., Timmers, C., Maiti, B., Saavedra, H.I., Sang, L., Chong, G.T., Nuckolls, F., Giangrande, P., Wright, F.A., Field, S.J., et al. (2001a). The E2F1-3 transcription factors are essential for cellular proliferation. Nature *414*, 457–462.

Wu, L., Timmers, C., Maiti, B., Saavedra, H.I., Sang, L., Chong, G.T., Nuckolls, F., Giangrande, P., Wright, F.A., Field, S.J., et al. (2001b). The E2F1-3 transcription factors are essential for cellular proliferation. Nature *414*, 457–462.

Wu, Z., Zheng, S., and Yu, Q. (2009). The E2F family and the role of E2F1 in apoptosis. Int. J. Biochem. Cell Biol. *41*, 2389–2397.

Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E., and Dyson, N.J. (1996). Tumor induction and tissue atrophy in mice lacking E2F-1. Cell *85*, 537–548.

Zamanian, M., and La Thangue, N.B. (1993). Transcriptional repression by the Rb-related protein p107. MBoC *4*, 389–396.

Zhang, J., and Jiao, J. (2015). Molecular Biomarkers for Embryonic and Adult Neural Stem Cell and Neurogenesis. Biomed Res Int *2015*.

Zhang, H.S., Gavin, M., Dahiya, A., Postigo, A.A., Ma, D., Luo, R.X., Harbour, J.W., and Dean, D.C. (2000). Exit from G1 and S Phase of the Cell Cycle Is Regulated by Repressor Complexes Containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell *101*, 79–89.

Zhu, L., Heuvel, S. van den, Helin, K., Fattaey, A., Ewen, M., Livingston, D., Dyson, N., and Harlow, E. (1993). Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes Dev. *7*, 1111–1125.

Zyskind, J.W., Wang, Y., Cho, G., Ting, J.H., Kolson, D.L., Lynch, D.R., and Jordan-Sciutto, K.L. (2015). E2F1 in neurons is cleaved by calpain in an NMDA receptor-dependent manner in a model of HIV-induced neurotoxicity. J. Neurochem. *132*, 742–755.