THE ROLE OF ACTIVATOR E2FS IN ADULT NEURAL STEM CELL QUIESCENCE AND ACTIVATION

By

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« Penser, ce n'est pas unifier, rendre familière
l'apparence sous le visage d'un grand principe.
Penser, c'est réapprendre à voir, diriger sa conscience,
faire de chaque image un lieu privilégié. »
ALBERT CAMUS, Le Mythe de Sisyphe

Abstract

Within the adult mammalian brain, Neural Stem Cell (NSC)s are maintained in distinct neurogenic niches in a mostly quiescent state. Activation of quiescent NSCs first requires re-entry into the cell cycle in order for the pool to proliferate and eventually commit to a neural fate, giving rise to newborn neurons. The canonical Retinoblastoma (Rb)-E2 Promoter Binding Factor (E2f) pathway is not only key in overcoming the Gap 1 Phase (G1)/S-phase restriction, but novelly appears to be involved in adult neurogenesis and NSC activation. I hypothesized that activator transcription factors E2 Promoter Binding Factor 1 (E2f1) and E2 Promoter Binding Factor 3 (E2f3) are crucial for exit from a quiescent state in adult NSCs. The contribution of the activator E2fs in this transition was studied using a Nestin-driven Cre Recombinase-Estrogen Receptor Tamoxifen-2 Ligand Binding Domain (Cre-ER^{T2}) system to induce targeted deletion of E2f1/3 within NSCs in adult mice. We show that loss of E2f1/3 causes significant neurogenic defects, including pro-neural activation and decreased pools of adult NSCs, that preferentially adopt a quiescent profile in the subventricular zone. We employed this model to further isolate subventricular zone-derived NSCs using a Rosa26:Yellow Fluorescent Protein (YFP) reporter and subsequently analysed transcriptional profiles by RNA sequencing. Loss of E2f1/3 shifts NSC transcriptomes towards one overlapping with quiescent neural stem cell signatures (Codega et al., 2014; Basak et al., 2018), further highlighting the requirement of these E2fs for initial activation. A significant portion of these differentially expressed genes are putative E2f

targets. Transcriptionally, major pathways involving cell metabolism, cellular signaling, and neural development are perturbed without activator E2f expression.

In effect, this combined approach based on *in vivo* data and bioinformatics analyses offers a method of prospective identification of novel regulators of adult neurogenesis that require the activator E2fs. Preliminary data suggests that AT-Hook Transcription Factor (Akna) is one such target worth pursuing. Cumulatively, this project describes a unique role for E2f1 and E2f3 during NSC exit from quiescence and subsequent activation towards differentiation. As ongoing maintenance of quiescent NSCs is a necessary prerequisite for lifelong neurogenesis, conclusions from this study could determine the therapeutic potential of targeting activator E2fs to combat the niche exhaustion associated with aging, injury, and neurodegenerative diseases.

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Contents

Glossary

aCSF Artificial Cerebrospinal Fluid 33

Akna AT-Hook Transcription Factor iv, 61, 63, 65, 72, 74, 76, 96

aNSC Active Neural Stem Cell 11, 12, 13, 14, 20, 29, 39, 54, 58, 59, 67, 69, 70

Ascl1 Achaete-Scute Homolog 1 15

bHLH Basic Helix-Loop-Helix 14, 15

Bmp Bone Morphogenic Protein 18

Bmp4 Bone Morphogenic Protein-4 17

BrdU 5-Bromo-2'-Deoxyuridine 2

Cacng1 Voltage-Dependent Calcium Channel Gamma-1 Subunit 61, 63

CBIA Cell Biology and Image Acquisition 33

Ccnd1 Cyclin D1 22, 23

CD133 Prominin 1 39, 66

Cdc6 Cell Division Control Protein 6 Homolog 61, 63

Cdk Cyclin-Dependent Kinase 23, 26, 74

Cdk4 Cyclin-Dependent Kinase 4 23

Cdk6 Cyclin-Dependent Kinase 6 23

cDNA Coding Deoxyribonucleic Acid 34, 35

ChIP Chromatin Immunoprecipication 35, 36, 48, 49, 61, 63, 64, 95

CIHR Canadian Institutes of Health Research vi

CKI Cyclin-Dependent Kinase Inhibitor 23

Cre-ER^{T2} Cre Recombinase-Estrogen Receptor Tamoxifen-2 Ligand Binding Domain iii, 30, 31, 96

CSF Cerebrospinal Fluid 9, 19

DAPI 4 ,6-Diamidino-2-Phenylindole 33, 40

mRNA Messenger Ribonucleic Acid 30, 38, 96

MyoD Myogenic Differentiation 1 27

Neo1 Neogenin 1 27

NES Normalized Enrichment Score 46, 47

NSC Neural Stem Cell iii, iv, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 26, 28, 29, 35, 37, 38, 39, 40, 41, 42, 44, 48, 49, 50, 51, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76

OB Olfactory Bulb 8, 9

p16 Cyclin-Dependent Kinase Inhibitor 2A 39, 67

PBS Phosphate-Buffered Saline 32, 33

PCA Principal Component Analysis 43

PCR Polymerase Chain Reaction 31, 34

PFA Paraformaldehyde 32

PGK-Neo Phosphoglycerine Kinase-Neomycin 5' 30

PPPARG Peroxisome Proliferator Activated Receptor Gamma 27

qNSC Quiescent Neural Stem Cell 11, 13, 14, 17, 20, 29, 39, 54, 58, 69, 70

qPCR Quantitative Polymerase Chain Reaction 34, 36

Rb Retinoblastoma iii, 20, 21, 22, 23, 25, 26, 28, 29, 37, 56, 70, 73, 76

RGC Radial Glial Cell 7

RMS Rostral Migratory Stream 8, 9

RNA Ribonucleic Acid 34, 35, 38, 42, 57, 58, 62

RT-qPCR Reverse Transcribed Quantitative Polymerase Chain Reaction 34, 38, 54, 57, 61, 62, 64, 70, 95, 96

SA-β-Gal Senescence-Associated β-Galactosidase 74

SGZ Subgranular Zone 3, 6, 7, 8, 9, 32, 66, 74

Shh Sonic Hedgehog 18

Sox2 Sex Determining Region Y-Box 2 10, 67

SVZ Subventricular Zone 3, 6, 7, 8, 9, 11, 12, 15, 16, 17, 28, 32, 33, 38, 39, 40, 41, 42, 61, 62, 64, 66, 67, 68, 71, 74, 76, 93

TAP Transit Amplifying Cell 9

TCAG The Centre for Applied Genomics 35

TSS Transcription Start Site 61, 63

WT Wild Type 64

YFP Yellow Fluorescent Protein iii, 30, 31, 33, 35, 38, 42, 62, 96

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Chapter 1

Introduction

1.1 Adult Neurogenesis

Key discoveries made in recent decades have overturned the once prevalent dogma that new neurons cannot be born within adult brains. In fact, neurogenesis – the generation of newborn neurons that arise from resident stem and progenitor pools and functionally integrate into the adult brain's circuits – is known to play crucial roles in memory and cognition throughout one's lifetime (Braun & Jessberger, 2014). This process becomes even more important within neurodegenerative contexts, disease states, and following injury.

1.1.1 An Overview of Adult Neurogenesis

Early studies investigating potential cell cycle progression within the brain predated the discovery of neurogenesis in humans by more than half a century. Deoxyribonucleic Acid (DNA) synthesis was unexpectedly observed in various cell types of the rodent brain (including neurons) and near sites of injury using radioactive thymidine incorporation assays (MESSIER et al., 1958; ALTMAN, 1962). These findings were complemented by later investigations of the primate Dentate Gyrus (DG), where certain cells had incorporated

5-Bromo-2'-Deoxyuridine (BrdU) (a thymidine nucleoside analog) into their DNA (Gould et al., 1998). Simultaneously, other regions of interest such as the striatum were being studied for their neurogenic potential within adult mice (Reynolds & Weiss, 1992). This accumulated knowledge led to the keystone discovery of adult neurogenesis within the human DG (Eriksson et al., 1998), effectively revolutionizing the field of neuroscience and giving birth to an exciting new branch of scientific enquiry.

Within the relatively short span of two decades, our understanding of neurogenic processes and their possible implications for human health has grown exponentially. Most notably, technical advances have enabled us to further supplement these initial observations and better characterize the scope of the new neurons' contributions. Among others, similar approaches have used either equivalent nucleotide incorporation assays or radioactive ^{14}C birthdating techniques to support the idea that new cells are being generated (Ernst et al., 2014; Spalding et al., 2013). Interestingly, the former study concludes that the turnover rates within the middle-aged human DG is equivalent to that seen within mice. Beyond these primary "gold standard" validations, other groups have generated supporting data in line with what is known. Using post-mortem human samples, neural progenitors were isolated and expanded *in vitro* (Palmer et al., 2001). Immunohistochemistry (IHC) analyses of similar samples further characterize changes in proliferation using markers of the neural lineage (Dennis et al., 2016). Taken together, these findings offer conclusive proof of the occurrence of neurogenesis and its relevance within adult human brains. Kempermann et al. have recently published an updated review on our current understanding of adult human neurogenesis and have posed questions that remain, for the time being, unanswered (Kempermann et al., 2018). Generally, one must allow for differences when translating knowledge from classical animal models to humans, or when evaluating why and how neurogenesis unfolds in specific

brain regions within the context of this animal.

Despite the considerable amount of knowledge accumulated thus far, there is still doubt cast by some related to the contributions and relevance of neurogenesis within the adult human brain. Recently, a pair of studies published within a month of each other took opposite stances on hippocampal neurogenesis: using similar approaches, one argued that the rate of neuron generation was negligible past childhood (Sorrells et al., 2018) whereas another maintained that the process was constant throughout time (Boldrini et al., 2018). Interestingly, this second paper acknowledges the decline through time in angiogenesis and neuroplasticity yet also insists that these core pools of differentiating and newborn neurons persist in healthy human brains as they age. Lastly, the group's findings shows a decline in the quiescent Neural Stem Cell (NSC) pool as being one the first changes observed within the human DG as it ages. The roles of these early quiescent cells and the mechanisms regulating their maintenance throughout a lifetime are topics at the core of this thesis. Moreover, despite differing opinions related to the actual neurogenic output of the brain, the fact that new cells are being generated (whatever their precise identities may be) and that they actively contribute to normal brain function cannot be denied.

1.1.2 Functional Significance of Neurogenesis

The identification of a dividing population of cells that gives rise to neurons within adult brains naturally led researchers to evaluate the functional importance of this continuous process in healthy adults, along with the multiple factors that modulate it. It is now generally accepted that two regions of the brain are constantly generating new neurons: the Subgranular Zone (SGZ) of the hippocampus's DG and the Subventricular Zone (SVZ) of the lateral ventricles (Ming & Song, 2005). The function of adult neurogenesis has been most strongly tied to memory and learning (Deng et al., 2010). The process not

only entails the generation of new neurons, but also their functional integration into local networks in or around these brain regions. Within the DG, the cells contribute to the animal's ability to map out and navigate through its surroundings (Hafting et al., 2005). Both structures are responsible for processing large amounts of sensory stimuli; however, individual stages of memory consolidation such as encoding or decoding are specific to either of the structures (Kesner, 2007). Experiments that manipulate the rates of neurogenesis in rodents demonstrated its importance in spatially-linked object recognition, pattern separation, and fear conditioning tasks (Jessberger et al., 2009; Clelland et al., 2009; Saxe et al., 2006). Given the fact that this process is restricted to such specific parts of the brain, and that many of these functions converge to offer a functional view that links memory and pattern separation with environmental sensing, some have even hypothesized that neurogenesis may be adaptively significant (Konefal et al., 2013). Somewhat paradoxically, though not incompatibly, others have suggested a role for adult neurogenesis in memory decay and forgetting (Gao et al., 2018; Frankland et al., 2013). Indeed, the generation of newborn neurons within the adult brain is crucial for maintaining the neural networks' ability to balance permanence and plasticity for lifelong memory formation (Lledo & Valley, 2016).

Beyond its involvement in memory, neurogenesis is also studied within the contexts of mood regulation and emotions. The heterogenous nature of major depressive disorder implies that a variety of causes may underlie this pathology: neurogenic disruptions are thought to be one such example (Sahay & Hen, 2007). Though an outright decrease in neurogenic rates does not guarantee depression-like symptoms (Jayatissa et al., 2010), these ideas are linked and supported by many correlative studies that highlight the beneficial effects of antidepressants on NSC generation (Park, 2019; Mahar et al., 2014). Anxiety and stress responses have many factors in common that interact and influence neurogenesis, and vice-versa (Anacker et al., 2018; Hill et al., 2015).

Taken together, these emotional contexts are translated not only into NSC output, but also into an overall modulation of synaptic plasticity, Long-Term Potentiation (LTP), and learning process efficiency (Schmidt-Hieber et al., 2004; Alam et al., 2018).

The list of regulators that influence the birth of new neurons is constantly being revised and added to, as novel connections to the niches and progenitor cells are made. Intrinsically, resident NSCs possess certain transcriptional pathways (many of which will be addressed in section 1.2.2) that instruct both fate and cell cycle decisions (Kageyama et al., 2019a). Alternatively, a panoply of external factors also exert their influences on this balance: neurotransmitters (Platel et al., 2010), hormones (Mahmoud et al., 2016), growth factors (Oliveira et al., 2013), antidepressants (Park, 2019), drug use (Suliman et al., 2018; Poulose et al., 2017), etc. Finally, lifestyle elements – such as running, cognitive training, or social isolation – are strong determinants of neurogenesis rates (van Praag et al., 1999; Zhang & So, 2019; Cinini et al., 2014). The interplay between all these variables is further predicated by either neurodegenerative disease or stroke (Yoneyama et al., 2011; Koh & Park, 2017). The reason for highlighting this catalogue of processes impacting neurogenesis is twofold: it sheds light on the impressive amount of signals being received and integrated by NSCs at any given time, and emphasizes the need to understand the basic mechanisms regulating NSC cell cycle and fate decisions. In this way, we will be better equipped to harness the therapeutic potential of adult NSCs in both healthy and injured cases, given the far-reaching implications on mental processes.

1.1.3 Adult Neurogenic Niches

Since the discovery of actively dividing cells within the brain, two things have been clear: that these cells are rare within the organ, compared to their postmitotic counterparts; and that they are only found in specific regions

therein. It was demonstrated that the hippocampus' SGZ and the lateral ventricles' SVZ were the most neurogenically active, becoming the field's classic (and best characterized) niches (Bonaguidi et al., 2012; Ponti et al., 2013). While some studies have flagged other brain regions as potentially giving rise to neurons within adulthood, their neurogenic output in normal conditions is negligible compared to the former two (Bernier et al., 2002; Magavi et al., 2000; Zhao et al., 2003; Evans et al., 2002). Developmentally, the earliest cells that could generally be described as NSC precursors are the neuroepithelial cells derived from the embryonic neural tube, following folding of the neural plate (Temple, 2001; Kriegstein & Alvarez-Buylla, 2009). While the scope of this thesis limits itself to adult NSC function, it is worth mentioning because the precise embryonic provenance of all subsequent adult NSCs is still being explored (Berg et al., 2019; Yuzwa et al., 2017). Slightly varying hypotheses have been proposed to explain the persistence of these neural precursors during a person's life. Briefly, the "sequential model" postulates that all neural precursors are formed during an embryonic boon preceding gliogenesis following birth (Kriegstein & Alvarez-Buylla, 2009). Likewise, the more recent "set aside model" illustrates tightly controlled populations of quiescent cells that are set apart from the actively dividing and expanding neural cells that make up the brain (Furutachi et al., 2015; Fuentealba et al., 2015).

The process undertaken by an embryonic neuroepithelial cell in becoming an adult NSC, or of an adult NSC awakening from dormancy to undergo terminal differentiation within the brain are enormously complex transformations that have both significant overlap and differences. Factors such as the developmental provenance of cells, their molecular markers, and especially the environmental cues offered by the niche are all crucial to contrasting and comparing adult NSCs and their potential for differentiation.

The Subgranular Zone

As mentioned previously, the embryonic neuroepithelial cells that make up the future spinal chord area further differentiate to give rise to self-renewing Radial Glial Cell (RGC)s that go on to populate the brain (Anthony et al., 2004). Beyond the generation of neuronal and astrocitic populations, small portions of these stem cells persist in the adult SGZ and SVZ as NSCs. Figure 1 may serve as a reference illustrating both locations of interest and the cellular subtypes found in these areas. Adult cells of the neural lineage vary slightly in terms of identity and dynamics based on their niche of origins. SGZ neurons originate from radial granule-like Type 1 cells. Once these begin to undergo fate specification and/or division, they progress to Type 2a/b intermediate progenitor cells. After successive rounds of divisions, the cells finally morph into Type 3 neuroblasts that must undergo the final stages of differentiation and maturation to become a proper hippocampal granule neuron (Anthony et al., 2004; Gebara et al., 2016). While this is a very high level overview of adult neurogenesis within the DG, it is important to keep in mind that many cellular characteristics are a reflection of overall cellular state: morphology, angle of cellular division, intracellular pathways, and expressed molecular markers to name a few (Abbott & Nigussie, 2020).

Naturally, this process of differentiation in the SGZ occurs within a broader landscape. As is the case for both niches, these cells are in close proximity with vasculature which has the potential to regulate neurogenic processes based on the animal's activity (Sawada et al., 2014). Moreover, while the mature neurons go through no significant migration and merely integrate within the adjacent granule cell layer, the extension of mossy fibres to the outer CA3 hippocampal regions is crucial in late cellular maturation (Faulkner et al., 2008; Zhao et al., 2006). This illustrates how the neurogenic output within adult brains is linked to key circuitry of the hippocampus.

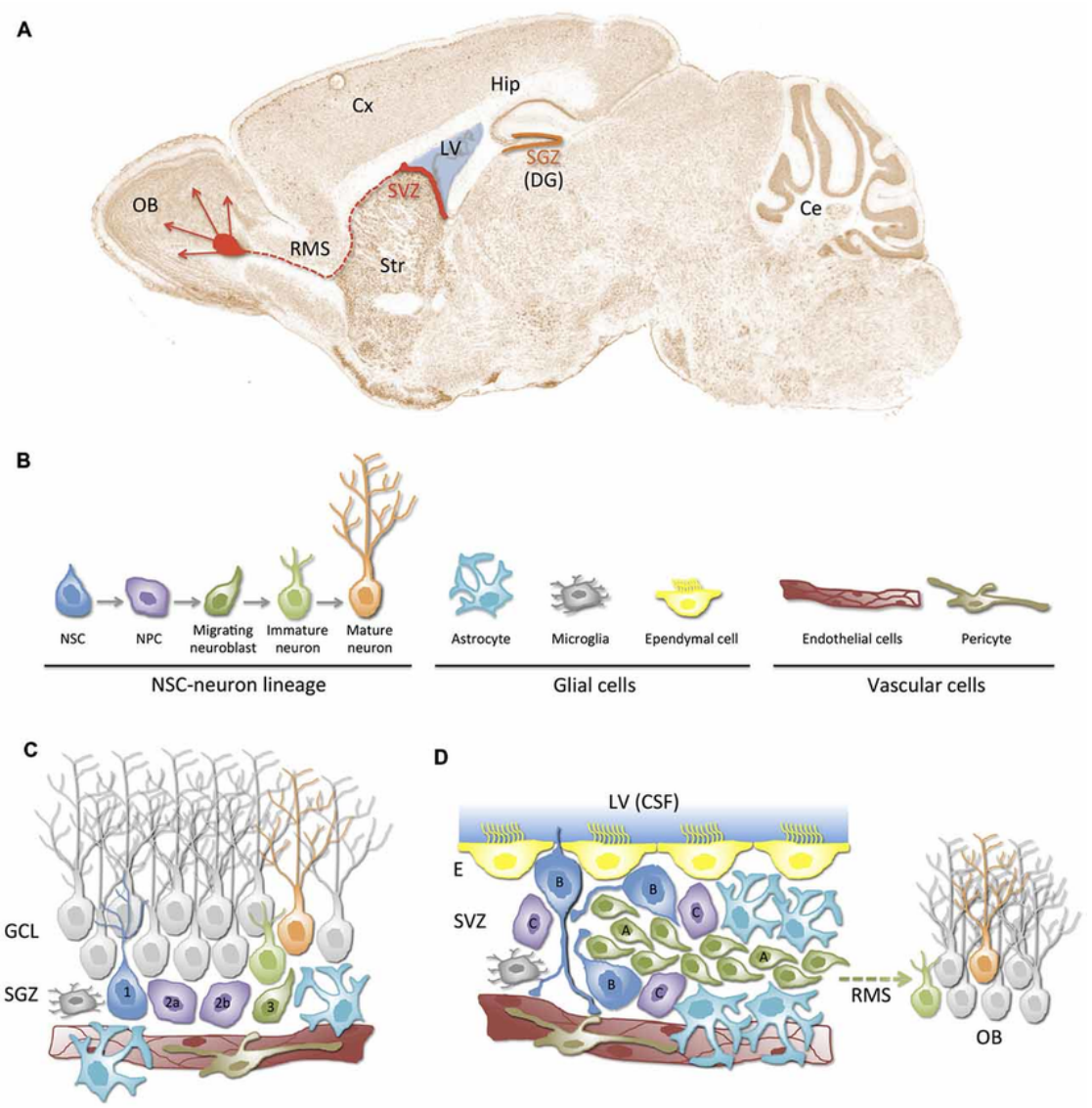

Figure 1: **Neurogenic niches and cellular subtypes found within the adult mouse brain**
(A) Saggital view of the brain with both the SGZ in the DG (orange) as well as the SVZ bordering the lateral ventricles (blue). Progeny originating from the latter zone migrate along the Rostral Migratory Stream (RMS) to integrate within the Olfactory Bulb (OB).
(B) Cell types found within the neurogenic niches may be grouped based on lineage: neural, glial, or vascular cells. Note that this generalized schema of differentiation from NSC to postmitotic neuron is common to both niches.
(C) Illustration of the SGZ niche.
(D) Illustration of the SVZ niche.
(Bátiz et al., 2015).

The Subventricular Zone

Neurogenesis within the SVZ of the lateral ventricles has key differences

from that of the hippocampus. These particularities should be kept in mind, as the bulk of the work in this thesis focuses on the SVZ. As illustrated in Figure 1, the ventricles' NSCs are called B1 cells and adopt characteristic pinwheel formations (Mirzadeh et al., 2008). It is necessary to keep in mind that although they may share some characteristics, these NSCs are distinct from the other astrocytes or ependymal cells of the zone (Doetsch et al., 1999; Doetsch et al., 1997). Similarly to the SGZ, location is important as this niche makes direct contact with both vasculature and the Cerebrospinal Fluid (CSF) with basal and apical elongations respectively (to be explored in Chapter 1.2.3). Morphologically, the cells possess a primary cilium through which they sense and integrate environmental cues. These B1 cells may cycle between states of quiescence and activation (Codega et al., 2014); through either homeostatic maintenance processes or following injury, these cells may be expanded and studied *in vitro* (Doetsch et al., 1999).

Following sufficient signaling, the B cells give rise to type C cells (Transit Amplifying Cell (TAP)s) that rapidly divide to generate type A immature neuroblasts. During these later stages of cellular differentiation and amplification, factors such as plane of division affect the balance between symmetric/asymmetric divisions which effectively modulate shifts towards either self-renewal or productive neurogenesis (Ponti et al., 2013; Noctor et al., 2008). Finally, these type A cells migrate with the help of astrocytes in groups called chains along the RMS to undergo final maturation and integrate within the OB (Doetsch & Alvarez-Buylla, 1996; Wichterle et al., 1997). Given differences between the species' reliance on olfactory senses, it is natural for there to be some differences between murine and human SVZ neurogenic outputs and functional integration (Sanai et al., 2004; Ernst et al., 2014; Sanai et al., 2011). The fact of the matter remains that the continuous generation of newborn adult neurons is crucial in both mammals' brains.

As previously mentioned, a large portion of the work done to establish the

field consisted in characterizing the molecular markers that may be used to discriminate between cells of the neural lineage and others, or even between cells at different stages of differentiation (Zhang & Jiao, 2015). Nowadays, multiple tools and models are at scientists' disposal to more precisely identify NSC populations and their eventual progeny within the adult (Semerci & Maletic-Savatic, 2016). Generally, combined expression based on a panel of molecular markers such as Nestin, Glial Fibrillary Acidic Protein (Gfap), and Sex Determining Region Y-Box 2 (Sox2) may be used to identify NSCs within both adult niches (Codega et al., 2014; Suzuki et al., 2010; Ellis et al., 2004) and discriminate between other similar cell types (e.g. astrocytes). Equivalent panels may be used to track cells from NSC to neuroblast to fully mature neuron. Given their integral role in the maintenance of neurogenesis throughout adulthood, the biology of this population of NSCs within the niches may be further explored to identify the factors that converge to trigger their activation and commitment to differentiation.

1.2 Neural Stem Cell Quiescence and Activation

Initial descriptions of a hypothesized state of quiescence were based on radioactive labelling experiments that characterized the distinct stages of the cell cycle (Steel, 1986). The key observation to come out of these studies was that not all cells seemed to divide at consistent rates, if at all. As scientists gained a better understanding of the cell cycle and its tightly regulated processes, the discovery of a restriction point in the process further added to the speculation that mechanisms existed that not only served to advance the cell cycle, but that could also effectively arrest it (Pardee, 1974). Sub-optimal environmental conditions (such as serum starvation or high cell to cell contact) were shown to also bring about this state of cellular exit (Stoker, 1972; Temin, 1971). Findings gradually led us to a generalized definition for cellular quiescence: a "reversible

growth/proliferation arrest … state induced by diverse anti-mitogenic signals" (Coller et al., 2006).

The reversibility of this state is indeed one of the key hallmarks of quiescence. Moreover, there is a natural overlap between the study of quiescence and stem cell biology: adult tissues are able to adapt or regenerate following various stimuli by awakening the dormant cells, thus offering the organism significant versatility (Cheung & Rando, 2013). Within this context, quiescent stem cells that re-enter cell cycle following stimulation are said to become active. At this point, it should be noted that there are various differences between an exit from cell cycle towards quiescence or towards senescence, mostly based on the damaging potential or potency of the signal (Khademi-Shirvan et al., 2020). The balance between niche replenishment and the production of committed cells following activation and expansion relies heavily on the cells' integration of intrinsic, extrinsic, and systemic signalling. As all downstream effects hinge on this initial process, the interplay between quiescence and activation (within NSCs and beyond) has far-reaching implications.

1.2.1 Characterization of the Cellular State of Quiescence

The subsequent sections focus purely on adult Quiescent Neural Stem Cell (qNSC)s and Active Neural Stem Cell (aNSC)s. While slight differences exist between these populations depending on the niches (including naming conventions), analysis will mostly focus on overall trends or defer to SVZ-based profiles. Given the fact that quiescence is sometimes associated with an overall dampening of cellular processes (e.g. metabolism, macromolecular processing and synthesis), this does not imply a state of generalized cellular passivity: on the contrary, it involves the recruitment of several robust cellular programs that jointly ensure the possibility of cell cycle re-entry and acts against differentiation (Cheung & Rando, 2013). Indeed, the setting aside of quiescent

NSCs within the SVZ has been shown to occur as early as mid-embryonic gestation in order to prevent the loss of these cells ahead of successive rounds of division and differentiation required during development (Fuentealba et al., 2015; Furutachi et al., 2015).

As discussed earlier, the key to quiescence is its reversibility within cells even though long stretches of time might have elapsed since they were last active. Studies with AraC have highlighted the robust resilience of these dormant adult NSCs (Doetsch et al., 1999). Nevertheless, immunolabelling and nucleotide analogue incorporation assays may be designed to detect a proportion of these lowly cycling cells (Codega et al., 2014; Urbán et al., 2016a; Cheung & Rando, 2013), though only up to a certain point (given the early entry into quiescence during development for certain pools of cells). Conversely, it is possible to evaluate the proportion of active NSCs by assessing Epidermal Growth Factor Receptor (Egfr) marker expression (Codega et al., 2014). These outlined methods serve to isolate general NSC groups and further characterize the distinct populations of active or dormant NSCs.

Technological advances of the recent decade have enabled scientists to explore these quiescent and active NSC populations at a much deeper level. The incorporation of transcriptome data offers a more nuanced view of these cellular identities, revealing a wealth of shifts that go beyond the modulation of cell cycle regulators and their transcriptional targets. For instance, one study used a fate mapping approach to not only demonstrate *in vivo* the long-term self-renewal capacities of aNSCs following a return to quiescence, but put forth a possible explanation for the regulation of NSC fate as being carried out by the SVZ niche (Basak et al., 2018). These approaches offer insight into differential metabolic profiles, wherein quiescence is associated with higher rates of glycolysis and lipid metabolism and activation is associated with a shift towards oxidative phosphorylation (Shin et al., 2015; Llorens-Bobadilla et al., 2015). Interestingly, the cells' transition towards activation is also associated

with an increase in protein synthesis, transcriptional priming, and a shift towards more neurogenic markers (Llorens-Bobadilla et al., 2015; Codega et al., 2014; Dulken et al., 2017). Meanwhile, cells that are quiescent also preferentially express transcripts that relate to cellular communication and contact between cells (Shin et al., 2015). Finally, these changes can also incorporate an epigenomic enhancing mechanism that helps enact quiescence in these cells (Martynoga et al., 2013). Figure 2 offers a succinct visual overview of most of these cellular characteristics that vary between qNSC and aNSC populations.

Taken together, it is important to go beyond the binary limitations drawn by each of these studies individually (because of the model of choice, the selected cellular population or marker, etc.) These findings describe a continuum through which adult NSCs can reversibly travel. While differences between these two populations remains anchored in the cells' status within the cell cycle, this model expands the cellular state and offers a holistic view that includes facets of metabolism, interactions with the environment, and internal cellular processes. Moreover, the various cellular states that NSCs can occupy introduces the notion of heterogeneity of quiescence. Factors that either contribute to these characteristics or modulate the balance of quiescence and activation will be the focus of the remainder of this section.

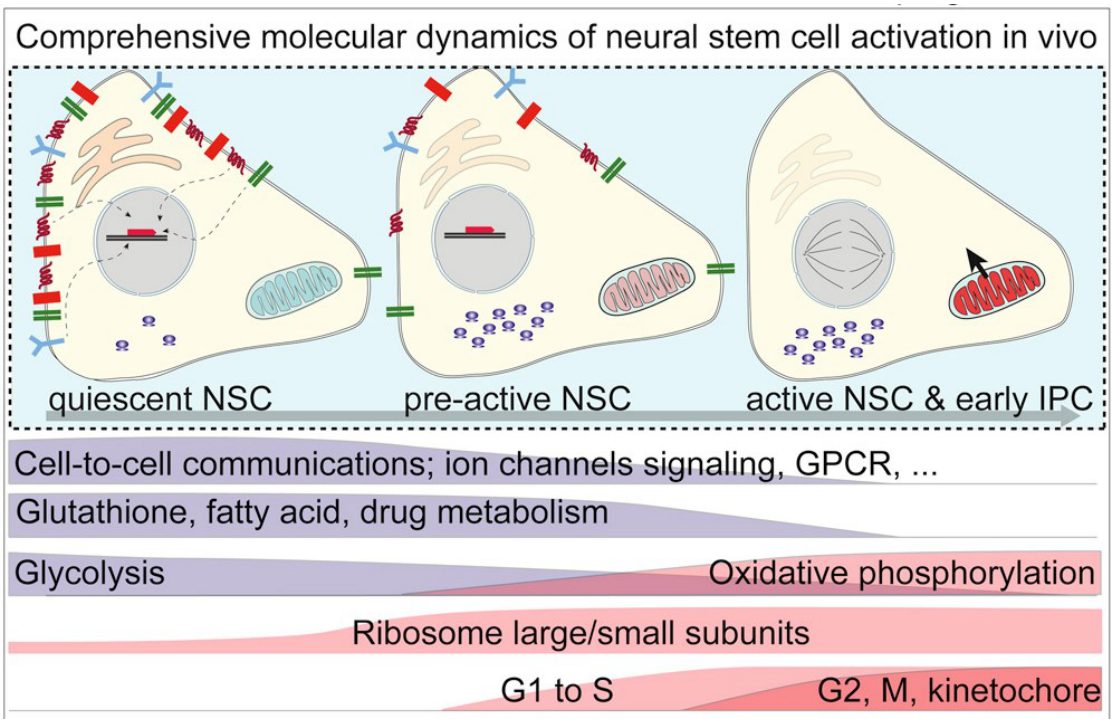

Figure 2: **Changes in molecular dynamics during the transition between quiescent and activated NSCs**
Overview of changes in molecular dynamics and cellular hallmarks specific to populations of qNSCs and aNSCs prior to differentiation, as revealed through single-cell transcriptome analysis.
(Shin et al., 2015).

1.2.2 Intrinsic Signals and Molecular Mechanisms of Quiescence

NSCs are exposed to countless signals in their milieu that may take various forms, either through soluble molecules, proteins, or direct contact. As has been previously discussed, qNSCs in particular express unique transcripts that enable them to better sense their environments and potentially shift their cellular states, as need be. However, these individual cells are responsible for integrating the wealth of signals to which they are exposed, and carrying out adjustments through intrinsic mechanisms and molecular pathways.

There exist a group of Basic Helix-Loop-Helix (bHLH) transcription factors that instruct for differentiation towards neural and astrocytic lineages within NSCs during development (Kageyama et al., 2019b). Working in an oscillatory

fashion and possessing the capability to either homodimerize or heterodimerize among each other, Achaete-Scute Homolog 1 (Ascl1) and Neurog2 are two such proneural factors (Imayoshi & Kageyama, 2014). Indeed, Ascl1 is of such crucial importance to neuronal commitment that these NSCs (without it and Math3, another proneural gene) cannot form certain neuroblast types during development (Tomita et al., 2000). Within the adult, NSCs that receive signals to activate first induce Ascl1 expression that in turn decreases cellular proliferation (Andersen et al., 2014). The consistent expression of Ascl1 is one of the first signals a cell carries out that can effectively shift the balance towards an active state. While this factor's proneural activity is well characterized by now (Castro et al., 2011), Ascl1 may also be degraded through destabilization by Huwe1. Thus, adult NSCs retain the capacity to return to a prior quiescent state (Urbán et al., 2016b). Additionally, Hes1 acts as a transcriptional repressor for these aforementioned bHLH factors (including for its own activity) (Takebayashi et al., 1994). Such fine-tuning of differentiation factors is afforded by Hes1 and Hes5 and has the possibility to pattern NSCs (Bansod et al., 2017). Though certainly not exhaustive, these examples of regulation through bHLH factors are crucial for the shift towards activation and eventual neural differentiation as they control broad proneural transcriptional programs.

Regardless of how these are initiated based on signals that the NSC has received, various signalling pathways help maintain or overcome the quiescent cellular state. For instance, Notch signalling was suggested to play a role in adult NSC maintenance within the SVZ in models where Rbpj inhibition was lifted, which led to generalized NSC activation and subsequent exhaustion of the pool (Imayoshi et al., 2010). The role of this signalling specifically in maintenance of activation in NSCs was further implied using a targeted model against Notch1 (Basak et al., 2012). Suffice it to say that, given the complexity of Notch signalling and its various receptors, these cascades nonetheless play

crucial roles in the maintenance of balance between actively held quiescent and (given the previous example) active state NSCs (Engler et al., 2018). Downstream of Notch2, Id4 was shown to be a significant promoter of NSC quiescence. Novelly, this study also acts as an example of the decoupling of adult NSC activation from subsequent neuronal commitment and differentiation (Zhang et al., 2019). Finally, Hedgehog signalling plays a role in shifting the balance between activation and quiescence in the SVZ (Daynac et al., 2016). Again, while states of both quiescence and activation involve multiple signalling pathways within cells, these recent findings illustrate the possible ways these changes are carried out: through direct repression or activation, or some combination thereof.

Finally, such basic cellular features as metabolic shifts and profiles may also enact changes in the state of quiescence, acting as much more than a readout of the cellular state. Master regulators such as FoxO3, in controlling oxygen metabolism, can also contribute to the long-term maintenance of NSCs (Renault et al., 2009). Strikingly, perturbation of lipogenesis pathways can also inhibit NSC proliferation, confirming that metabolism can go beyond reflecting cellular states of quiescence and activation, but also directly contributes to these balance shifts (Knobloch et al., 2013). Finally, it has been shown that enhancing the lysosomal pathway in quiescent NSCs can partially enhance their ability to re-enter quiescence, an ability that typically declines with ageing (Leeman et al., 2018).

Taken together, it becomes evident that many mechanisms are at play during both quiescence or activation: transcriptional programs, cellular pathways, and metabolism are all downstream processes that can be modulated according to stimuli or contacts sensed by the cell. Conditions such as ageing or various disease states all have their own effects on both cellular processes and neurogenic outputs. While these topics go beyond the scope of this thesis, they further reinforce the need to understand the basic mechanisms regulating

quiescence and activation in order to ensure lifelong NSC persistence in adults.

1.2.3 Extrinsic Niche Signals

As previously mentioned, qNSCs in particular are specifically primed to enhance such features as cell communications and receptors enabling them to intercept any signals from their environments. Though there are a wide array, extrinsic signals from the extracellular environment are inevitably translated into signals that are propagated throughout the NSC. The following section outlines such environmental cues, whose subsequent cellular effects may or may not involve mechanisms from the previous section.

The adult SVZ, as illustrated in Figure 1, is a highly organized microenvironment with many different cell types and elements in close proximity to each other. Among others, the extracellular matrix of the SVZ is composed of structures called fractones. Not only do they support and modulate the area's NSCs and other cells by capturing growth factors (Kerever et al., 2007), but they also sequester Bone Morphogenic Protein-4 (Bmp4) and present it to the NSCs, effectively inhibiting proliferation (Mercier & Douet, 2014). Moreover, other niche-derived cues associated with the matrix act on the NSCs in a way that maintains quiescence (Porcheri et al., 2014). Similarly, NSC exposure to most neurotransmitters is shown to have a variety of effects on cellular proliferation and overall neurogenesis (Berg et al., 2013). Given their quiescent state, NSCs express a range of neurotransmitter receptors and can be impacted by both direct and indirect routes (Giachino et al., 2014). As an example, mossy cells support NSC quiescence through direct (glutamatergic) and indirect (GABAergic) neurotransmitter signaling (Yeh et al., 2018). As such, cells within close range of the NSCs can sway or support their maintenance of quiescence, either through direct physical contact or else through neurotransmitter release in proximity.

It is simple to state that the elements with which the NSCs are in close

contact significantly mould their cellular states. However, other cells at a range of the niches also regulate NSC function through the release of signalling molecules and growth factors. Many of these pathways are introduced in section 1.2.2. The role of these cues are now mostly understood, as they have broad effects on stem cell activity within tissues. Broadly speaking, Bone Morphogenic Protein (Bmp) signaling contributes to the maintenance of quiescence within NSCs (Johnston & Lim, 2010). While these Bmp factors may be generated by the NSCs themselves, they're also secreted by the zone's granule neurons, and their antagonists have been shown to promote NSC proliferation (Mira et al., 2010). Similarly, the Wnt proteins have long been regarded as key players in general stem cell control, especially as it relates to the creation of a niche environment capable of supporting both self-renewal and expansion of these cellular numbers (Clevers et al., 2014; Qu et al., 2013). In this case, Wnts are secreted once again by the NSCs themselves, as well as the niche astrocytes (Lie et al., 2005). Finally, Sonic Hedgehog (Shh) is produced by neurons at a distance from the niche itself, yet has a demonstrated effect on NSC establishment of quiescence and proliferation (Noguchi et al., 2019; Ahn & Joyner, 2005). While these potent morphogens have broad effects on many stem cell populations, it is relevant to note that these secreted factors may find their origins in both NSCs and many of the cells within the general neurogenic areas (Camacho-Aguilar & Warmflash, 2020). Such molecules and other growth factors aside (Delgado et al., 2014), Milk Fat Globule-EFG Factor 8 (Mfge8) (also known as lactadherin) is an example of a protein that acts, in an either paracrine or autocrine fashion, on NSCs in the milieu to both maintain quiescence and support long-term neurogenesis within the niches (Zhou et al., 2018; Okazaki & Gotoh, 2018).

Given the systems that were reviewed in this section, it is relevant to note that the bulk of these examples consist of local endogenous cues (stemming from the niche and its multiple components) that interact to actively maintain

NSC quiescence. While this state of quiescence is generally active by default in healthy organisms, this balance can be perturbed by signals received that are separate from the niche environments, and reflect changes in the person or mouse's physiological state.

1.2.4 Systemic Contributions and Considerations

In this context, the elements that come into contact with the NSCs in the niches become especially relevant: the vasculature, the CSF, and any other cues derived from the brain itself (Horowitz et al., 2020; Gato et al., 2020; Zappaterra & Lehtinen, 2012). Here, messengers (whether or not they are of the same classes as the ones described in the previous sections) are transported towards the neurogenic niches. In this sense, the adult NSCs have access to a much wider range of information about the organism's current behaviour and physiology. As always, these are taken into account in the individual cells' integration and maintenance of quiescence or activation. Briefly, there are many factors that have been shown to sway neurogenesis at a high level: physical exercise and voluntary running, stress, inflammation, nutrition and diet, depression and antidepressants, learning and certain mental processes, etc. (Saraulli et al., 2017; Lucassen et al., 2015; Hoeijmakers et al., 2014; Micheli et al., 2018; Cameron & Glover, 2015) In some cases, it is possible to determine a partial mechanisms of action, or else study the specific feature of neurogenesis that is being modulated (e.g. exit from quiescence, NSC proliferation, differentiation). However, given the complex interactions at play when taking into account behavioural or lifestyle changes, it is difficult to specifically assess the impact on quiescence or activation in adulthood.

In sum, it is clear that even when considering the full developmental journey from nascent NSC to fully differentiated adult neuron, appropriate attention must be paid to the early mechanisms that regulate activation and return to quiescence. Whether focusing on the cellular level, the niche itself, or

the organism as a whole, one must appreciate the multitude of interrelated processes that combine and contribute to the dynamic status of qNSCs or aNSCs at any given moment. Thus, long-term maintenance of a pool of quiescent NSCs in adulthood is only possible through the proper orchestration of qNSC and aNSC dynamics and fates.

1.3 The Rb/E2f Pathway

Generally, topics discussed thus far have related to the characterization of cellular states of NSCs as they relate to the early stages of commitment towards productive neurogenesis and local stem cell maintenance within the adult brain. Given this context, to talk about cellular identity and differentiation necessarily brings to mind the underlying machinery common to all cells that drives cell cycle progression and mitosis. As will be demonstrated in this final section, the pocket proteins and the E2 Promoter Binding Factor (E2f) transcription factors may be seen as two sides of the same coin. While countless influences converge to direct the fine balance between NSC quiescence and activation, this interplay is further nuanced by taking into account the E2fs' roles in the cell cycle, stemness, and neural development.

1.3.1 Cell Cycle and the Canonical Rb/E2f Pathway

Initial research on the Retinoblastoma (Rb) gene led to the discovery and characterization of the first tumor suppressor element within the human genome (Benedict et al., 1983; Cavenee et al., 1983). Clinically, mutations in the Rb gene cause a recessive, heritable cancer that originates in the patient's retina – hence the name – and most commonly affects young children (Dimaras & Corson, 2019). Interestingly, the reintroduction of a single copy of the gene was sufficient to counteract the oncogenic effect of this mutation (Huang et al., 1988). Initially, the E2fs were shown to be transcriptional activators for a class

of viral promoters through binding to specific DNA sequences (Kovesdi et al., 1987). While findings quickly accumulated and expanded the functional scope of both of these genes, the seminal discovery that conclusively married the two factors was published in 1992 (Nevins, 1992). This laid the groundwork for the current understanding of the Rb/E2f complex interaction and function, wherein cell cycle progression (and concurrent transcription of early Gap 1 Phase (G1) genes) is dependent on the alleviation of the Rb-induced block. An illustration of the key interactions between Rb and the E2fs as well as their involvement during early cell cycle progression between Go and G1 is shown in Figure 3.

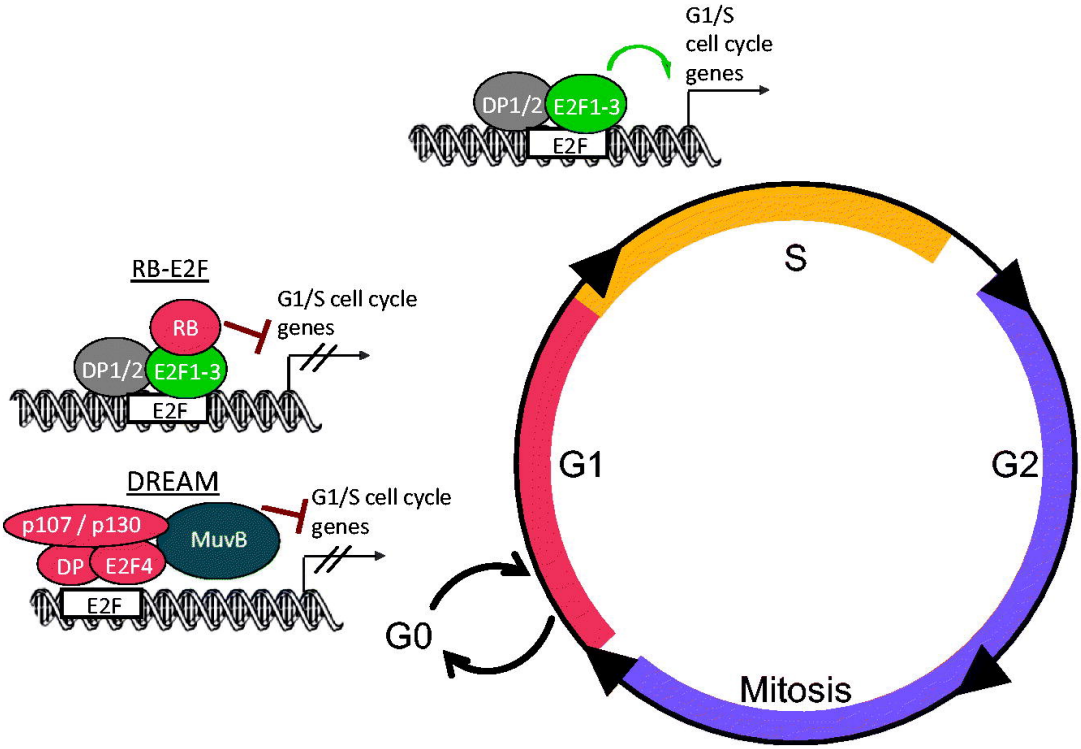

Figure 3: **Early cell cycle progression as it relates to the Rb/E2f complex** Schematic illustrating the major players that drive cell cycle transcriptional control up to the G1/S phase checkpoint. While pocket proteins and the E2fs are at the core of this regulation, they act in tandem with various components of the Dimerization Partner, Rb-like, E2f, And Multi-Vulval Class B (DREAM) complex. Shifts in transcriptional activity are denoted by the arrows. Modified from (Fischer & Müller, 2017b).

Rb is part of a family of factors known as the pocket proteins (Giacinti & Giordano, 2006). Along with p107 and p130, these three proteins are grouped together because they possess a conserved binding pocket region in which they may bind to the E2fs, among others. Similarly to Rb, all three are capable of blocking the cell cycle ahead of the S phase (Dyson, 1998). While the finer discrepancies that differentiate the proteins from one another may be overlooked in this study to maintain a focus on the E2fs, it is relevant to mention that they independently play crucial roles in stem cells (Mushtaq et al., 2016), neural development (Slack & Miller, 1996), and neurogenesis (Ferguson & Slack, 2001). Otherwise, there exist three Dimerization Partner (DP)s that heterodimerize with certain E2fs during both transcriptionally active and repressive cellular states (Komori et al., 2018). Finally, MuvB is a distinct, 5 component complex that acts uniquely as a transcriptional repressor and has particular requirements for association with pocket proteins and E2fs (Fischer & Müller, 2017a). While many of these factors come into play at many stages of cellular division or withdrawal, their contributions are here most relevant within the context of preparation for G1 entry.

In essence, Rb and E2f activity and status within the cell determine whether or not it begins the process of division. The E2fs drive early cell cycle entry because they bind to the DNA and enable transcription of specific targets related to cell cycle regulation and preparation for DNA synthesis. Association with Rb reverses these effects and holds the block on progression. Rb exerts a secondary level of control on transcription by recruiting the aforementioned DREAM complex that further inhibits transcription (Giacinti & Giordano, 2006) – these last 3 states find themselves illustrated in Figure 3. Crucially, Rb binding to the E2fs is regulated through its phosphorylation status: hypophosphorylation enables binding to E2f, whereas hyperphosphorylation relieves this transcriptional impasse. Initially, the phosphorylation onto Rb is carried out by a combination of cyclins (Cyclin D1 (Ccnd1)) and their respective

Cyclin-Dependent Kinase (Cdk)s (Cyclin-Dependent Kinase 4 (Cdk4) and Cyclin-Dependent Kinase 6 (Cdk6)) as a downstream response to external mitogenic signaling (Goel et al., 2018). Cyclin-Dependent Kinase Inhibitor (CKI)s offer an additional level of upstream control, acting on Cdks (Lim & Kaldis, 2013). Though this is a simplistic view of how many ambient signals converge to shift the balance between Rb- or E2f-mediated stasis or progression, E2f-driven transcription of these early stage genes also further complexify the situation. For instance, some of the first changes seen following Rb release and E2f and increases in E2 Promoter Binding Factor 1 (E2f1) and Ccnd1, which circles back and creates a positive feedback loop and starts to ready the cell for approach of the first checkpoint (Schafer, 1998). While the dynamics at play leading up to and following the Rb/E2f-determined tipping point are complex, the central and interrelated role of these two deciding factors should be kept in mind.

1.3.2 The E2f Transcription Factors

Given the decades that have elapsed since their discovery, and the location they hold at the heart of both regular and aberrant cell cycle functioning, the E2fs have been studied quite broadly. Currently, there are 8 known genes that may give rise to 10 individual proteins in mammals (Kent & Leone, 2019). A summation of the E2f proteins and an illustration of their functional domains is shown in Figure 4. Keeping with the context of their initial characterization, they are commonly grouped based on their transcriptional influence: E2f1 to E2 Promoter Binding Factor 3 (E2f3) are transcriptional activators, and E2 Promoter Binding Factor 4 (E2f4) to E2f6 are transcriptional repressors. E2f7 and E2f8, given their lack or transactivation and pocket protein binding domains, may interchangeably be classified as atypical or repressor E2fs. Moreover, given the variable expression of E2f3 isoforms in quiescent or proliferating cell types, E2f3b is sometimes considered a repressor (Leone et al.,

2000). However, these binary differences do not always hold up in all circumstances and must not be overstated. For the sake of simplicity, subsequent mentions of the activator E2fs will refer to E2f1 to E2f3 inclusively.

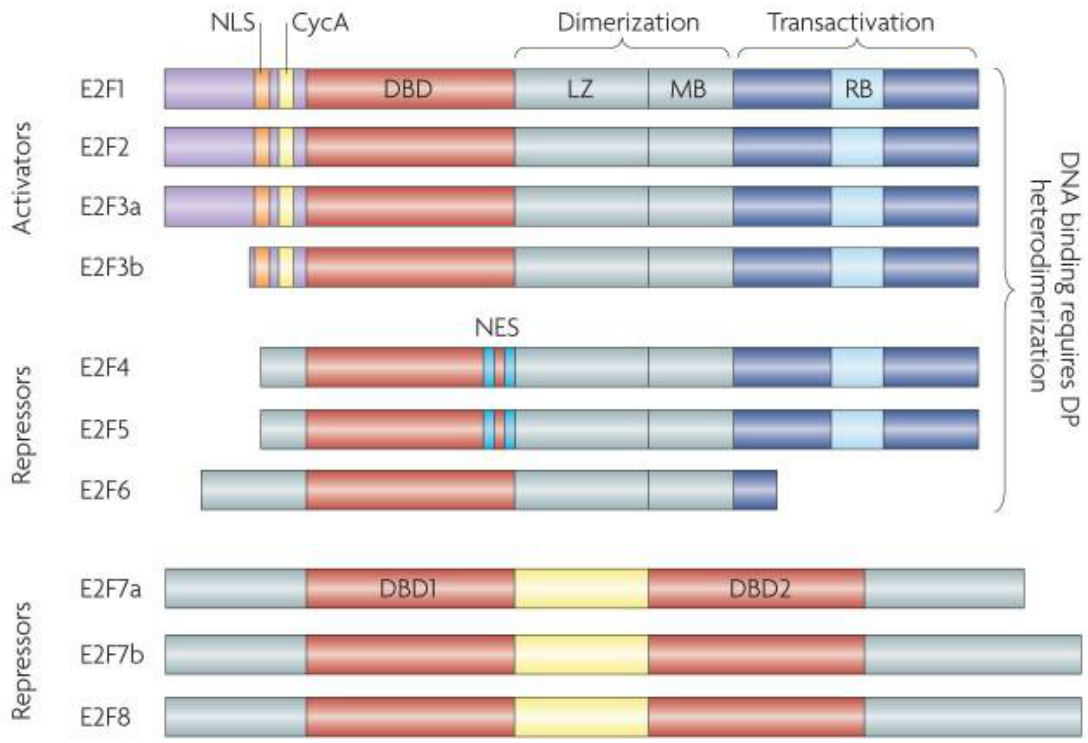

Figure 4: **Domains of the E2f family member proteins**
Schematic identifying the key domains that are characteristic to each member of the E2fs' individual functions.
(Chen et al., 2009).

Besides the large number of different factors, their study is made much more complex by the generalized overlap in binding across many tissues, and the fact that they often exhibit functional redundancy between activators and inhibitors (Xu et al., 2007; Attwooll et al., 2004). Also, a feature of their functions within cells, the E2fs can also act antagonistically (between activators and repressors) following experimental modifications, and modulate their own (or others') expression through feedback loops across all members (Frolov et al., 2001; Christensen et al., 2005). While these factors must be acknowledged at first, limiting ourselves to the key activators allows for a more focused study of

the topic.

The Activator E2Fs

The subclass of E2fs that classically support transcription and drive cell cycle will be the focus of this project. Initially, single mutant models allowed us to methodically compare the effects following ablation of a single transcription factor. Moreover, in limiting the scope to E2fs1-3, variables such as DP and pocket protein are further simplified as they can only complex with Rb and are necessarily expressed in the cell cycle (Dimova & Dyson, 2005; Wu et al., 2001). Broadly speaking, the perturbation of any single activator E2f mostly translates to some proliferative defect in the mouse's tissues (Humbert et al., 2000; Field et al., 1996). In establishing the models, loss of E2f3 translated to a significant reduction in expected genotype frequency and suggested a key role in development. Mutant embryonic fibroblasts from this strain proliferated less, and downstream target transcription was abrogated in key players; nonetheless cellular immortalization was still possible. In the case of E2f1 deficiency, many aspects of development and physiology were remarkably unaffected though some tissues also showed aberrant proliferation. Interestingly, loss of specific E2fs throughout the animals can lead to unique defects based on the factor in question. For instance, E2f1 knockout mice also demonstrated defects in the thymus and apoptosis (the latter being a better studied feature of E2f1 in particular) (Stanelle & Pützer, 2006).

Subsequently, knockout models combining the loss of various activator E2fs were generated and bred. In these, the requirement for E2f3 during development was heavily implied as most of these models were not viable (Wu et al., 2001). Working once again with embryonic fibroblasts, this team offered substantial *in vitro* data that confirmed that while a triple knockout fully eliminates the potential for proliferation, combinatory assays demonstrated an effect on cell cycle target transcription yet cells remained viable (albeit in a

slowed proliferative state). While loss of a single E2f seems to be compensated by the remaining members, it is relevant to note that combining knockouts (especially between E2f1/3) can greatly sway phenotypes. While this is a generalized overview of loss of function murine characterizations, the subsequent sections dealing with cells of either stem or neural lineages offer more in depth views on specific features.

1.3.3 The Role of E2fs in Stem Cells

Given the methods through which they drive cell cycle progression, it is natural to approach the E2fs' contributions to stem cell fate direction using the lens of transcriptional control (Julian & Blais, 2015). Generally, stem cells lie at the heart of both embryonic and adult tissues and are necessarily involved during development, tissue homeostasis, and even tumorigenic transformation (Wang, 2019). A large body of knowledge now exists (generated using loss of function models for the pocket proteins or the E2fs) that illustrates the crucial roles of E2fs during cellular proliferation, differentiation, survival, and fate specification (Sage, 2012). First, differentiation and cell cycle stage are tightly linked: G1 phase cells in particular are primed for differentiation (Sela et al., 2012). Within NSCs and progenitors, balance between proliferation and differentiation is regulated by an upstream Cdk-mediated switch (Lim & Kaldis, 2012). Upon commitment to differentiation, these cells also adjust relative lengths of their G1 and S phases (Arai et al., 2011). Indeed, the "cell cycle length hypothesis" – which postulates that time spent in G1 phase is said to be correlated with a increase in sensitization to some differentiation cues – has been studied in NSCs and seems to involve the machinery acting upon the balance between Rb and the E2fs (Lange & Calegari, 2010). These findings are further nuanced when one considers the shift in the E2fs' classical role as activators to that of repressors in actively differentiating cells (Chong et al., 2009b). While these examples illustrate the importance of the E2fs in cell

cycle-dependent control of stem cells, the pocket proteins and factors have also been shown to shape cellular identity using separate mechanisms. For instance, these may direct cellular proliferation, fate commitment, and maintenance of pluripotency without modulation of classic cellular dynamics (Chong et al., 2009a; Kareta et al., 2015; Vanderluit et al., 2007).

Much of the potency of the E2fs relate to their ubiquitous nature and their functions as transcriptional regulators. The advent of modern methods to assess binding profiles of individual factors enabled researchers to conduct large-scale assays on multiple E2fs, using a variety of cell lines and tissues (Lee et al., 2011; Asp et al., 2009; Julian et al., 2016; Xu et al., 2007). Briefly, this E2f-led modulation of stemness and fate genes is carried out through targeting specific genes (that may or may not be involved in cell cycle progression). For instance, the E2fs modulate key factors that influence differentiation in various ways: Myogenic Differentiation 1 (MyoD) in myoblasts (Asp et al., 2009), Peroxisome Proliferator Activated Receptor Gamma (PPPARG) in adipocytes (Fajas et al., 2002), and Neogenin 1 (Neo1) during neurogenesis (Andrusiak et al., 2011). Beyond previously explored links to general stem cell maintenance, the factors also sway cellular survival (Conklin et al., 2012) and death (Julian et al., 2008; Carnevale et al., 2012) through targets of apoptotic pathways. Finally, it is possible to describe metabolic regulation through E2f targeting, which has already been demonstrated as being an important fate determinant (Folmes et al., 2013; Ochocki & Simon, 2013). As such, the role of the E2fs is as deeply entrenched in cell cycle progression as it is in a multitude of pathways that converge to modify regulation of states of stemness. The remaining section will limit its analysis of the E2fs as they relate to neural development.

1.3.4 The Role of E2fs in Neural Development and Neurogenesis

Examples of the E2fs' contributions to neural development and neurogenesis have been previously incorporated into the text as they relate to cell cycle control and regulation of stemness. However, the cellular machinery comprised of the E2fs (and the necessary pocket protein counterparts) has distinct influences on key neurogenic features such as the quiescence/activation balance between NSCs, commitment to differentiation, and eventual fate of these cells. For instance, beyond its requirement during development, maintenance of Rb has been shown to be paramount to normal progenitor proliferation and long-term survival of adult-born neuroblasts (Vandenbosch et al., 2016). Portions of this study were corroborated and confirmed within the SVZ, where long-term neuroblast survival (following an initial boon within the adult) was curtailed by a subsequent increase in apoptosis (Naser et al., 2016). Interestingly, this last study notes that the changes in question occur without any shifts in NSC self-renewal capacity. Generally, it has also been established that the Rb/E2f axis plays a deciding role in late differentiation and neuronal migration (Andrusiak et al., 2011; Ghanem et al., 2012).

However, these factors' roles in NSC quiescence and activation remain incompletely characterized. Given the earlier studies, Rb alone does not seem to be the definitive arbiter that acts to maintain cellular quiescence in adult stem cells (Walkley & Orkin, 2006). Conversely, there exists various pieces of data that establish the overall role of the activator E2fs in adult neurogenesis, including contributions to overall NSC pools and self-renewal (Julian et al., 2013; Cooper-Kuhn et al., 2002; Yoshikawa, 2000). This convergence between the subset of the E2f family, neurogenesis, and cell cycle regulation make up the crux of the current study.

1.4 Rationale and Hypothesis

Considering the topics of both NSC quiescence and the expansive roles of the activator E2fs beyond cell cycle – in either neural development and stem cell identity – it is possible to make a strong case to investigate the role of E2f1 and E2f3 and their effects upon this distinct cellular state in NSCs. In either position, qNSCs and aNSCs are stably poised by active cellular programs that have the potential to shift dynamically in accordance with signals from the neurogenic niches, the brain itself, or available systemic cues. Similarly, cellular stasis or cycling is achieved through the interplay between Rb and the activator E2fs. The shifts in balance one way or the other for both models, in the long term, essentially determine the fate of the NSCs within the whole of the adult niches. The maintenance of these cells' numbers and capacity to activate as required is paramount to overall health, or in cases of injury and neurodegeneration.

As such, given their demonstrated importance in both cell cycle control and stem cell identity, **I hypothesize that the activator transcription factors E2f1 and E2f3 are required for exit from quiescence in adult NSCs.** In order to evaluate this question, two aims have been chosen: first, *to characterize the effects following E2f1/3 loss within adult NSCs at the molecular and cellular level*; and second, *to study the requirement of E2f1/3 for exit from quiescence, focusing on molecular signature changes of the NSC population*.

Chapter 2

Materials and Methods

2.1 Mice

Two previously characterized mouse lines were crossed and used to establish the colony that generated all experimental animals. Transgenic mice lacking E2f1, generously gifted by Dr. Michael E. Greenberg through Dr. David S. Park (Field et al., 1996), were bred with mutant E2f3$^{F/F}$ mice, generously gifted by Dr. Gustavo Leone (Wu et al., 2001). In the Greenberg model, the E2f1 gene was modified through insertion of a Phosphoglycerine Kinase-Neomycin 5' (PGK-Neo) cassette within exon 3 and the deletion of exon 4, effectively abrogating E2f1 Messenger Ribonucleic Acid (mRNA) expression in all cells. Similarly, E2f3 transcript expression was modified through excision of the full third exon using a Nestin-Cre Recombinase-Estrogen Receptor Tamoxifen-2 Ligand Binding Domain (Cre-ER^{T2}) mouse model from Dr. Suzanne J. Baker (St. Jude Children's Research Hospital) (Chow et al., 2008). This was achieved by inserting Locus of Crossover (loxP) restriction sites flanking the genomic sequence of interest. As such, the Cre recombinase in the presence of tamoxifen permanently excises this portion of the E2f3 gene in cells expressing nestin and generates a non-functional E2f3 protein. Moreover, Yellow Fluorescent Protein (YFP) expression (at least one allele of which is required in experimental mice)

from Rosa26-YFP reporter mice is also controlled by this Cre-ER^{T2} system. The resulting transgenic mice were maintained on a mixed genetic background (a combination of C57BL/6, FVB/N, and S129) (Charles River Laboratories, Wilmington, MA).

All experiments were carried out comparing E2f1/3 Heterozygous (EHT) mice possessing one functional, wild-type allele of both E2f1 and E2f3 (E2f1$^{+/-}$ E2f3$^{F/+}$ Nestin-Cre-ER$^{T2+/-}$) against E2f1/3 Knockout (EKO) mice homozygous for mutant E2f1 and E2f3 genes (E2f1$^{-/-}$ E2f3$^{F/F}$ Nestin-Cre-ER$^{T2+/-}$). EHT mice were generated by breeding an EKO mouse with a C57BL/6 wild-type mouse and keeping offspring with the required genotypes. Similarly, EKO mice were generated by breeding together two EKO mice (one of which carried one allele for the Cre recombinase) and keeping offspring with the required genotypes. Both male and female mice were used indiscriminately when composing experimental replicate groups. Throughout the breeding process, mice were genotyped using the REDExtract-N-Amp Tissue Polymerase Chain Reaction (PCR) Kit (Sigma-Aldrich) following the manufacturer's instructions along with primers for the 4 modified transgenes (Table 9).

This model generates a fusion protein Cre recombinase that also carries a modified ligand to the human estrogen receptor. As such, exposure to tamoxifen causes activation of the Cre within targeted cell populations, enabling both deletion of E2f3 and transcription of the YFP reporter. Both EHT and EKO adult animals aged 8 to 12 weeks were administered with a dose of TAM equivalent to 200 mg/kg for 5 consecutive days, similar to what has been previously published with equivalent Nestin-Cre-ER^{T2} models (Khacho et al., 2016).

2.2 Perfusion and Cryosectioning

Following Intraperitoneal (IP) Euthanyl injection, experimental mice were perfused transcardially with 25 mL 0.9% cold saline, followed by 25 mL freshly prepared, chilled 4% Paraformaldehyde (PFA) (pH 7.4). Brains were then dissected normally from skull, and post-fixed overnight at 4°C in vials containing fresh 4% PFA. The following day, brains were washed with 1% Phosphate-Buffered Saline (PBS) and then transferred to vials containing a solution of 20% sucrose, 0.03% sodium azide, and 1% PBS at 4°C for at least 48 hours, until brains are no longer floating.

Prior to cryosectioning, the prepared brains were frozen for 1 minute in isopentane chilled to -35°C after which they were mounted onto chucks and coated with Tissue-Tek OCT Compound (Sakura). Working at a temperature of -22°C, serial coronal sections of the tissue were cut at 30 μm thickness on a cryostat (Leica) at the University of Ottawa Louise Pelletier Histology Core. Tissue sections of the SVZ and SGZ were collected in wells containing a solution of 0.01% sodium azide and 1% PBS. These free-floating sections were stored at 4°C in this solution until ready to proceed to IHC staining.

2.3 Immunohistochemistry, Imaging and Quantification

For each stain, a subset of fixed floating tissue sections were washed 3 times with a solution of 1% PBS, then incubated with primary antibodies (Table 8) in a solution of 0.1% Tween20, 0.1% Triton X-100, and 1% PBS for 24 hours at 4°C. After primary incubation, tissue was similarly washed with a solution of 1% PBS and incubated with respective secondary antibodies (Table 8) in a solution of 0.1% Tween20, 0.1% Triton X-100, and 1% PBS for 2 to 4 hours at room temperature. This step additionally incorporates staining with

4 ,6-Diamidino-2-Phenylindole (DAPI) at 1 μg/mL (Sigma-Aldrich). Finally, stained tissue sections were washed one last time with 1% PBS, dried on Superfrost Plus Slides (Thermo Fisher) and fixed with Immuno Mount (GeneTex). Prepared slides were stored at 4°C and protected from light until imaging.

All slides were imaged using the Zeiss LSM800 confocal microscope at the University of Ottawa Cell Biology and Image Acquisition (CBIA) Core. SVZ sections were captured at 20x magnification with tiling and using 3 Z-stacks spanning 10 μm. Images were processed after the fact with a SUM projection in Fiji (Schindelin et al., 2012).

All cells lining the SVZ niche were counted manually using Fiji software. Total counts of all imaged sections were then tallied and multiplied by 9 (the number of wells in which the brain was serially divided during cryosectioning), to offer a representative estimation of such cell populations per structure.

2.4 Flow Cytometry and RNA Isolation

Following IP Euthanyl injection, brains were removed from the adult mice and further dissected to isolate tissue of the SVZ while working in fresh, chilled Artificial Cerebrospinal Fluid (aCSF). Between 3 to 5 brains were combined to generate a single experimental replicate. Tissue was processed with both mechanical and enzymatic digestions using scalpel blades and a solution of Papain (Cedarlane Labs), respectively. The sample was then triturated to form a single cell suspension and cleaned using a Percoll gradient (Sigma-Aldrich). These samples were sorted using the MoFlo Astrios EQ cell sorter (Beckman Coulter) at the University of Ottawa Flow Cytometry and Virometry Core Facility. Only cells that demonstrated YFP expression were isolated and kept for experimental purposes. Purified cell samples were then pelleted and stored frozen at -80°C until other technical replicates were ready for subsequent

processing in parallel.

Ribonucleic Acid (RNA) from accumulated sorted cell pellets was isolated using the PicoPure RNA Isolation Kit (Thermo Fisher) in conjunction with the RNAse-Free DNAse Set (Qiagen), following instructions from the manufacturers. Eluted RNA purity was immediately assessed with a NanoDrop (Thermo Fisher) and stored at -80°C for downstream applications.

2.5 RT-qPCR

In order to maximize the amount of Reverse Transcribed Quantitative Polymerase Chain Reaction (RT-qPCR) validations that could be run on all samples, previously purified RNA was first converted to Coding Deoxyribonucleic Acid (cDNA) with the SuperScript IV VILO Master Mix (Invitrogen). Using 20 ng of RNA per sample reaction, cDNA was generated using a Thermocycler according to the manufacturer's amplification protocol. Subsequently, expression levels of candidate genes were measured by Quantitative Polymerase Chain Reaction (qPCR) using the PerfeCTa SYBR Green FastMix Reaction Mix (QuantaBio). Primers for each candidate gene were used at a final concentration of 300 nM (Table 9). The manufacturer's Fast 3-Step Cycling protocol was modified using the RotorGene Q System (Qiagen) as follows: initial denaturation at 95°C for 30 seconds; a repeating PCR cycle of 95°C for 5 seconds, 60°C for 15 seconds, and 72°C for 10 seconds; followed by a melting curve ranging from 60 to 95°C with a one degree increase per second. Anywhere from 30 to 40 amplification cycles were performed depending on the individual amplification curve for each candidate gene. Gene expression differences between experimental genotypes were determined by normalizing to Glyceraldehyde 3-Phosphate Dehydrogenase (Gapdh) expression within the same sample. All analyses were carried out using the $\Delta\Delta$ Ct method correcting for efficiency and in compliance with Minimum Information for Publication of

Quantitative Real-Time PCR Experiments (MIQE) standards and guidelines (Bustin et al., 2008).

Gene expression on Chromatin Immunoprecipication (ChIP) samples (detailed below) did not require amplification and conversion to cDNA. The same Reaction Mix, Fast 3-Step Cycling protocol and Rotor Gene Q System outlined previously were used. However, primers for each candidate gene were used at a final concentration of 500 nM (Table 9). Data are presented as a percentage of enrichment relative to non-normalized IgG binding values.

2.6 RNA Sequencing and Bioinformatics

RNA aliquots isolated from YFP^+ cells – 3 biological replicates of each EHT and EKO genotype – were sent to The Centre for Applied Genomics (TCAG) (Toronto, Canada) for library preparation and sequencing. Prior to sequencing, quality of RNA samples was assured with Agilent 2100 Bioanalyzer 2100. In preparation for analysis, FastQ files received from TCAG were aligned to the mouse genome (mm10) to generate BAM format files using HISAT (Kim et al., 2015). Genes were assigned to raw sequence reads using featureCounts (Liao et al., 2014) and overall differential gene expression was analyzed using DESeq2 (Love et al., 2014). A list of genes whose expression is affected by E2f1 and E2f3 knockout in adult NSCs was generated with a padj < 0.05 and a log2 fold-change cut-off of 0.5.

Gene Ontology (GO) analysis of these differentially expressed genes was performed using g:Profiler (Reimand et al., 2016). Individually, ordered query analyses were run for both upregulated and downregulated genes relevant to Biological Processes (GO:BP). Gene Set Enrichment Analysis (GSEA) was run with similar parameters on these two gene lists with software from the Broad Institute at the Massachusetts Institute of Technology (Subramanian et al., 2005). Finally, an Enrichment Map was generated with combined data from the original ordered GO analyses using Cytoscape (Merico et al., 2010; Shannon

et al., 2003).

2.7 ChIP

ChIP assays were performed based on a previously published protocol (Liu et al., 2010). Briefly, adult neurospheres cultured from wild-type C57BL/6 mice 8 to 12 weeks of age were amplified to achieve sufficient chromatin yields for the assay, being passaged no more than 3 times. From previous experience, initial cross-linking with 11% formaldehyde carried out for 10 minutes at room temperature was optimal for cultured adult neurosphere cells. Chromatin was sheared to appropriately sized fragments using a probe sonicator, cycling through a protocol of 1 second on and 4 seconds off 150 times. Individual ChIP samples were prepared with 0.8 μg of selected antibody and 10 μg of prepared chromatin. ChIPs were done with antibodies against IgG, E2f1, E2f3, and E2f4 (Table 8). Eluted chromatin was purified using a phenol:chloroform:isoamyl alcohol extraction and MaXtract High Density tubes (Qiagen). The resulting Genomic Deoxyribunucleic Acid (gDNA) pellets were resuspended in water and directly assessed with qPCR and genomic primers (Table 9).

Chapter 3

Results

3.1 *In vivo* Characterization of the SVZ Following Loss of E2f1/3

While disruptions in individual activator E2fs can have a wide range of effects on neurogenesis (Cooper-Kuhn et al., 2002; Julian et al., 2013), the factors' ability to autoregulate and functionally compensate for one another had to be circumvented by targeting multiple members (Bracken et al., 2004; Kong et al., 2007). The generation of a model in which E2f1 transcript expression was fully abrogated and E2f3 expression could be specifically targeted within cells of the brain (neatly bypassing multiple E2f knockout embryonic lethality) enabled us to first study the generalized effect of loss of activator E2fs on neurogenesis in the adult brain. This project builds in parallel on work previously published within our lab. Briefly, the Rb protein (studied beyond the context of cell cycle and E2f complexing) was revealed to be critical for normal cortical development and maintenance of adult neuronal populations (Vandenbosch et al., 2016). Additionally, it is now established that E2f3's dual isoforms individually influence specific NSC fate decisions by altering Sox2 gene expression (Julian et al., 2013). However, these experiments were carried out with transgenic E2f3 mouse models distinct from the one in this study.

Initial characterizations with the combined E2f1/3 mutant model only achieved full knockout for both genes during adulthood, following induction with tamoxifen. Thus, mRNA transcript expression was significantly reduced for both genes in cells of the brain expressing Nestin, compared to heterozygous controls (Figure 5). Due to the lack of antibodies that could adequately discriminate individual E2f family members, it was impossible to validate by blotting for proteins. However, as is evidenced throughout the following experiments, the loss of activator E2fs is further confirmed through various "direct" (i.e. transcript sequencing data) and indirect (i.e. phenotypic changes, transcriptional signature shifts) methods.

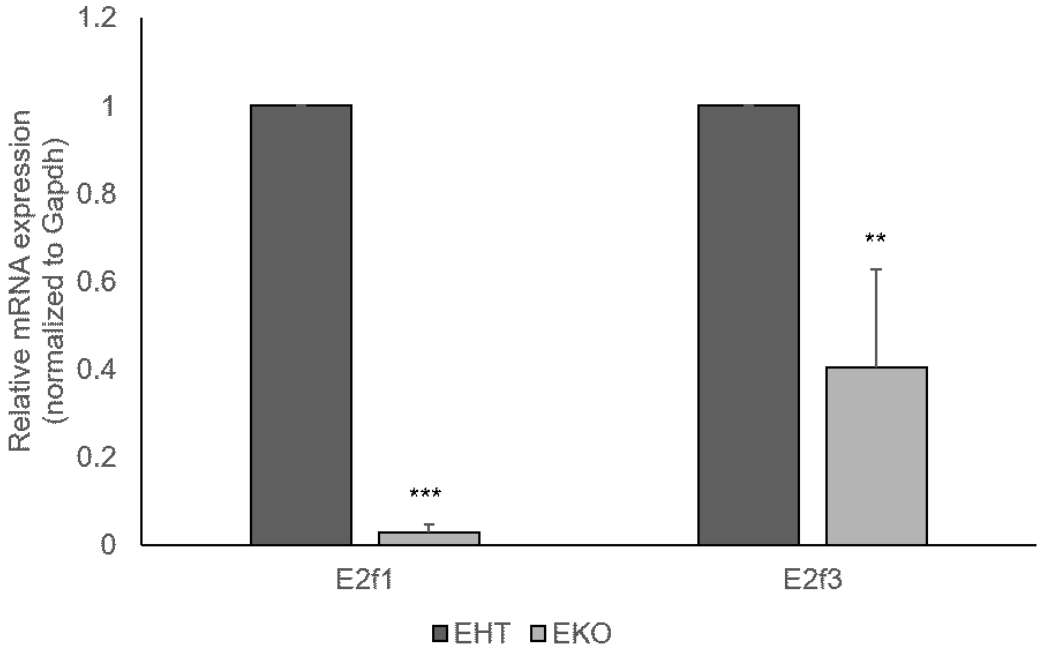

Figure 5: **Confirmation of activator E2f1 and E2f3 knockout**
RT-qPCR from RNA extracted from adult NSCs. Tissue dissected 10 Days Post-Injection (DPI) from the SVZ, followed by YFP^+ cell isolation with Fluorescence-Activated Cell Sorting (FACS). n = 3, ** $p < 0.01$, *** $p < 0.001$, two-tailed T-Test. Error bars show standard deviation.

Initial observations surveyed the impact of loss of activator E2fs on both adult neurogenic niches. Briefly, it was demonstrated through in-depth IHC analysis that knockout impairs overall neurogenesis, pro-neural activation, and

NSC pool numbers in both areas of the brain. These data may be found in a previous lab member's thesis (Yakubovich, 2019). Already, the loss of activator E2fs in both neurogenic niches suggests that they play a crucial role during neural development *in vivo*, beyond their previously established involvement with the cellular machinery.

As it was determined that gross neurogenic output was severely hindered without them, we wondered specifically at the factors' roles in adult NSCs, being the cells that eventually generate all other newborn neurons. In 2014, Codega et al. proposed a purification method to isolate qNSCs and aNSCs from the adult murine SVZ. These populations demonstrated cell cycle kinetics, neurogenic capabilities, stem cell properties, and molecular signatures that built up a picture of generalized quiescent or activated states. We repeated this method using the E2f1/3 transgenic line to evaluate the effect of knockout on these two NSC populations. An IHC stain of Gfap, Prominin 1 (CD133), and Egfr designated $CD133^{+}/Gfap^{+}/Egfr^{-}$ cells as qNSCs and $CD133^{+}/Gfap^{+}/Egfr^{+}$ cells as aNSCs. The proportion of activated NSCs in EKO animals was halved to roughly 12% 4 weeks after knockdown and 8% 8 weeks after knockdown. Comparatively, roughly 22% of all NSCs identified in the adult ventricle with this method were active (Figure 6). Intuitively, this reduction in activation of NSCs following E2f1/3 loss might have been caused by an increase in cellular quiescence. Cells temporarily withdraw from the cell cycle during quiescence, so Cyclin-Dependent Kinase Inhibitor 2A (p16) expression was used to assess this cellular state (Terzi et al., 2016). As such, the number of stem-like cells of the SVZ exiting cell cycle 4 weeks after knockdown is almost double that of an EHT animal (Figure 7). Taken together, these data offer a more complete view of the mechanisms behind this *in vivo* reduction in neurogenesis following loss of activator E2fs. In this model, adult NSCs exhibit decreased activation as per Egfr expression and increased cell cycle exit of stem-like progenitors.

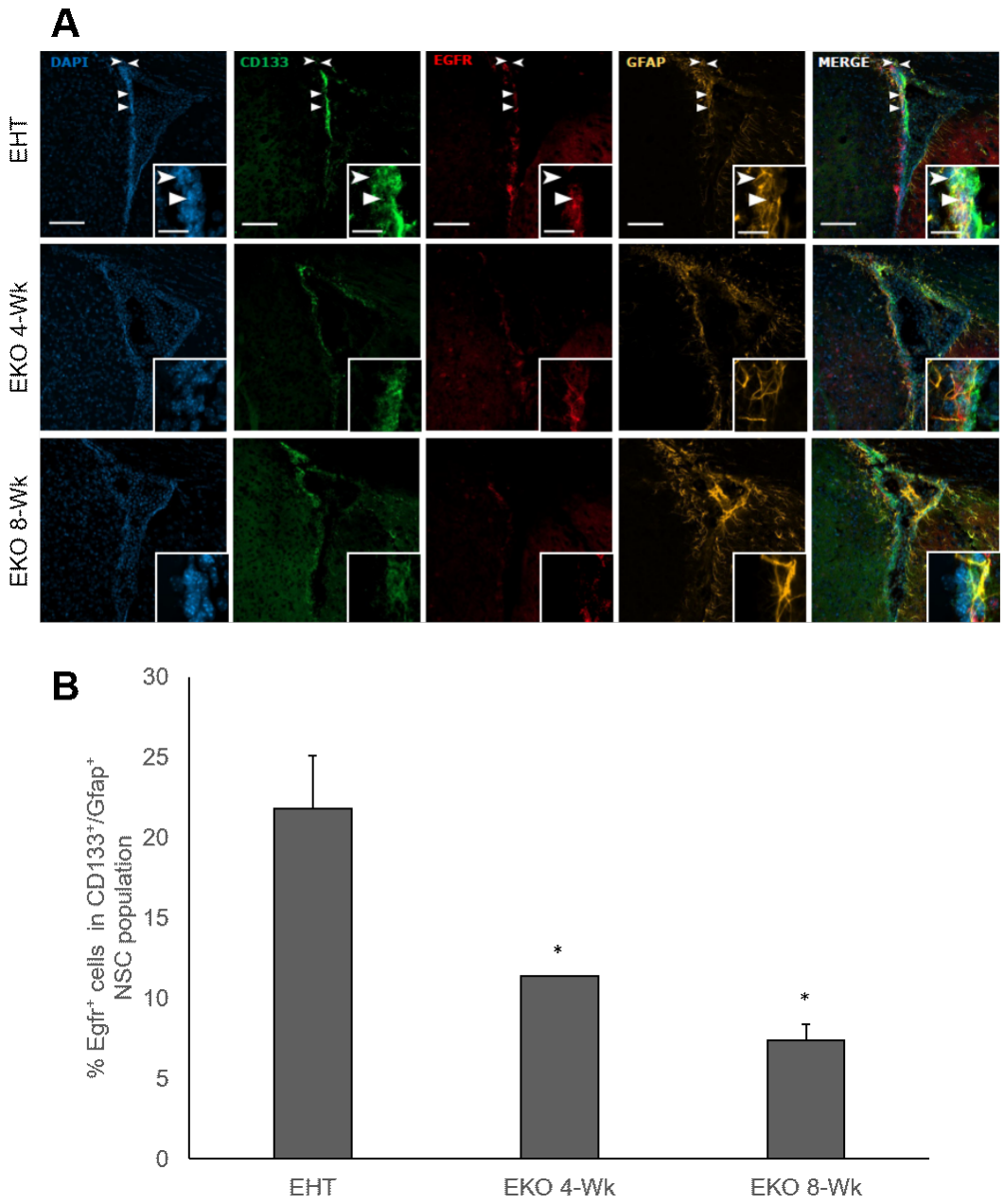

Figure 6: **E2f1/3 knockout reduces activation of adult NSCs in the SVZ**
(A) Representative confocal images of nuclei ($DAPI^+$), stem and ependymal cells ($CD133^+$), activated NSCs ($Egfr^+$), NSCs and astrocytes ($Gfap^+$) in the dorsolateral region of the adult SVZ. Conditions shown are either at 4 weeks (EHT and EKO 4-Wk) or 8 weeks (EKO 8-Wk) post-injection. Notched arrowheads show quiescent NSCs ($CD133^+/Gfap^+/Egfr^-$), whereas filled arrowheads show activated NSCs ($CD133^+/Gfap^+/Egfr^+$). Insets are 63x views.
(B) Quantification of active NSCs as a fraction of total NSCs in the adult SVZ. Conditions shown are either at 4 weeks (EHT and EKO 4-Wk) or 8 weeks (EKO 8-Wk) post-injection. n = 3 for EHT and EKO 4-Wk, n = 5 for EKO 8-Wk, * $p < 0.05$, two-tailed T-test and two-tailed two sample unequal variance T-Test, respectively. Error bars shown as standard error.
Work done by E. Yakubovich.

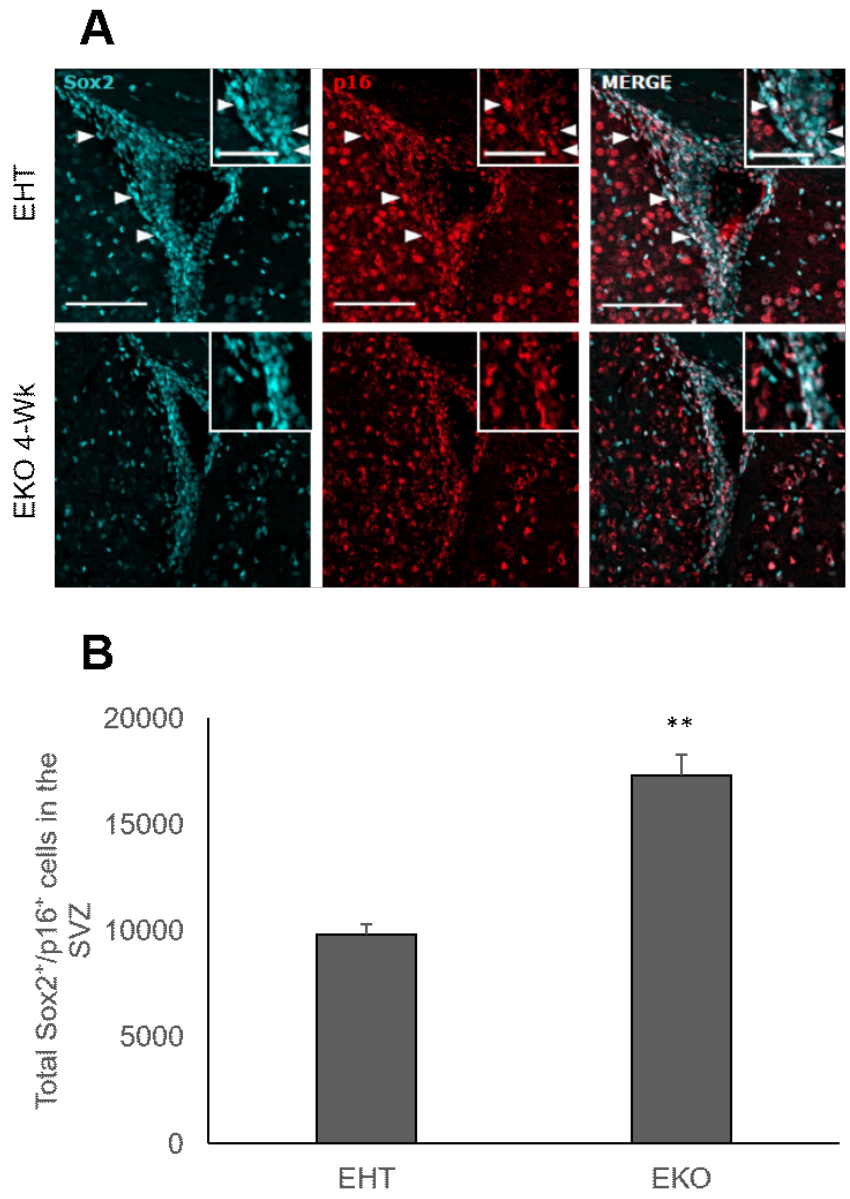

Figure 7: **E2f1/3 knockout increases cell cycle exit in adult NSCs in the SVZ**
(A) Representative confocal images of NSCs ($Sox2^+$) and cells undergoing cell cycle exit ($p16^+$) in the dorsolateral region of the adult SVZ. Conditions shown are at 4 weeks (EHT and EKO 4-Wks) post-injection. Arrowheads show cells that are withdrawing from the cell cycle ($Sox2^+/p16^+$). Insets are 20x views.
(B) Quantification of double-labelled $Sox2^+/p16^+$ cells in the adult SVZ. Conditions shown are at 4 weeks (EHT and EKO 4-Wk) post-injection. n = 3, ** $p < 0.01$, two-tailed T-test. Error bars shown as standard error.
Work done by E. Yakubovich.

3.2 Characterization of Transcriptional Changes Following Loss of E2f1/3

Given our initial characterization of the neurogenic output of the SVZ and the relative effects on NSC populations, we then aimed to characterize the global transcriptional changes following E2f1/3 knockdown in adult NSCs. Bulk RNA samples from YFP^+ EHT and EKO cell populations were isolated and used to generate libraries destined for RNA Sequencing (RNAseq). The quality of the dataset was assured by comparing clustering of 3 biological replicates per experimental genotype and filtering expression data for most significant reads based on padj and log2 fold-changes (Figure 8, Chapter 2.6). Of the 21,378 measurable genes, 4,229 showed significant transcriptional modifications representing 2,102 upregulated genes and 2,127 downregulated genes.

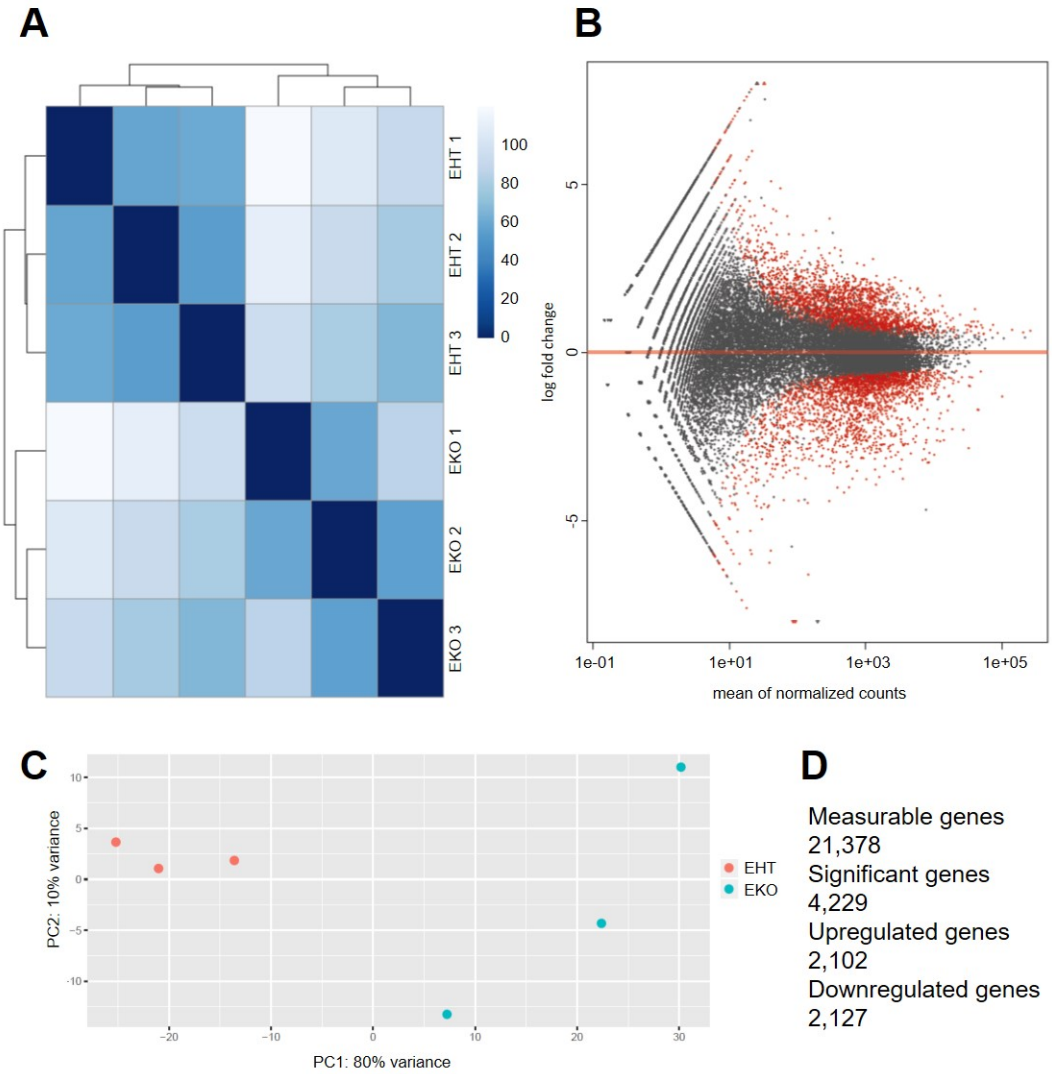

Figure 8: **Overview of E2f1/3 knockout RNAseq gene expression dataset of adult NSCs from the SVZ**
(A) Heatmaps comparing sample-to-sample distances with hierarchical clustering to compare all E2f1/3 EHT and EKO samples.
(B) MA-plots of log2 fold changes in relationship with the mean of normalized counts. Points in red have an adjusted p-value of less than 0.05.
(C) Principal Component Analysis (PCA) plots show samples in a 2D plane and determine the principal axes of variation.
(D) Summary of total measurable genes, significant genes, upregulated and downregulated genes from MA-plots.

In order to gain insight into general transcriptional trends when comparing EHT and EKO cells, g:Profiler was used to generate a list of biological process terms based on both upregulated and downregulated gene lists. The first 10 classifications for each category are listed in Table 1. Generally, upregulated

transcripts belonged to classifications related to the cytoskeletal architecture and microtubule network of a cell. Conversely, downregulated transcripts belonged to cell cycle, cellular division, and organelle organization groupings. These underexpressed groupings are unsurprising, given the fact that the E2f1/3 model removes two key cell cycle activators. What's more, the downregulation of cell cycle and cell cycle processes gene ontologies serves as an indirect confirmation of the quality of the RNAseq data set. GSEA yielded similar results for both sets of upredulated and downregulated gene lists (Table 2). The most upregulated gene sets were related to cytoskeletal components. Interestingly, cilium assembly and dynamics were highlighted throughout. Also, hallmark E2f targets was the most significantly enriched gene set following E2f1/3 knockdown, along with other cell cycle and mitotic processes. Enrichment plots for the most enriched gene set in both upregulated and downregulated transcript lists are shown as examples in Figure 9. In sum, overall transcriptomics data for adult EHT and EKO NSCs mainly highlights the dampening of the cellular E2f machinery as it relates to cell cycle progression and to a lesser extent (based on p-values) the increase in ciliary dynamics. Notably, pathways for neurogenesis or NSC identity are not immediately obvious, compared to *in vivo* data from Chapter 3.1.

Table 1: **Individual gene ontology analysis of upregulated and downregulated genes with E2f1/3 knockout in adult NSCs**

Upregulated in EKO Gene	p-value	Downregulated in EKO Gene	p-value
Cilium organization	1.97^{-21}	Cell cycle	6.03^{-59}
Cilium assembly	2.28^{-21}	Cell cycle process	1.42^{-55}
Cilium movement	6.02^{-21}	Miotic cell cycle	6.95^{-54}
Plasma membrane bound cell projection assembly	6.03^{-20}	Mitotic cell cycle process	1.03^{-47}
Cell projection assembly	2.13^{-18}	Cell division	3.52^{-40}
Axoneme assembly	1.76^{-14}	Chromosome organization	5.93^{-39}
Microtubule bundle formation	6.99^{-11}	Chromosome segregation	6.06^{-35}
Cell projection organization	5.21^{-8}	Nucleic acid metabolic process	2.58^{-33}
Plasma membrane bound cell projection organization	4.09^{-7}	Cellular component organization	2.29^{-29}
Movement of cell or subcellular component	1.23^{-6}	Organelle organization	3.44^{-29}

Gene ontology analysis for biological processes run with gProfiler on differentially expressed transcripts in E2f1/3 knockout RNAseq. First 10 classifications shown for each set of genes.

Table 2: **Gene set enrichment analysis of upregulated and downregulated genes with E2f1/3 knockout in adult NSCs**

Upregulated in EKO		Downregulated in EKO	
Gene	**NES**	**Gene**	**NES**
Cilium movement	2.38	Hallmark E2f targets	-2.35
Axoneme assembly	2.36	Chromosome, centromeric region	-2.32
Motile cilium	2.24	Hallmark G2M checkpoint	-2.30
Cilium assembly	2.20	Cell cycle, mitotic reactome	-2.29
Axonemal dynein complex assembly	2.19	Mitotic prometaphase reactome	-2.28
Cilium organization	2.18	Chromosome segregation	-2.28
Axoneme	2.18	Mitotic cell cycle	-2.28
Axoneme part	2.17	Chromosomal region	-2.27
Dynein complex	2.17	Mitotic cell cycle progress	-2.27
Cilliary plasm	2.13	Cell cycle reactome	-2.26

Ordered query GSEA run with Broad Institute software on differentially expressed transcripts in E2f1/3 knockout RNAseq. Sorted by normalized Normalized Enrichment Score (NES). First 10 classifications shown for each set of genes.

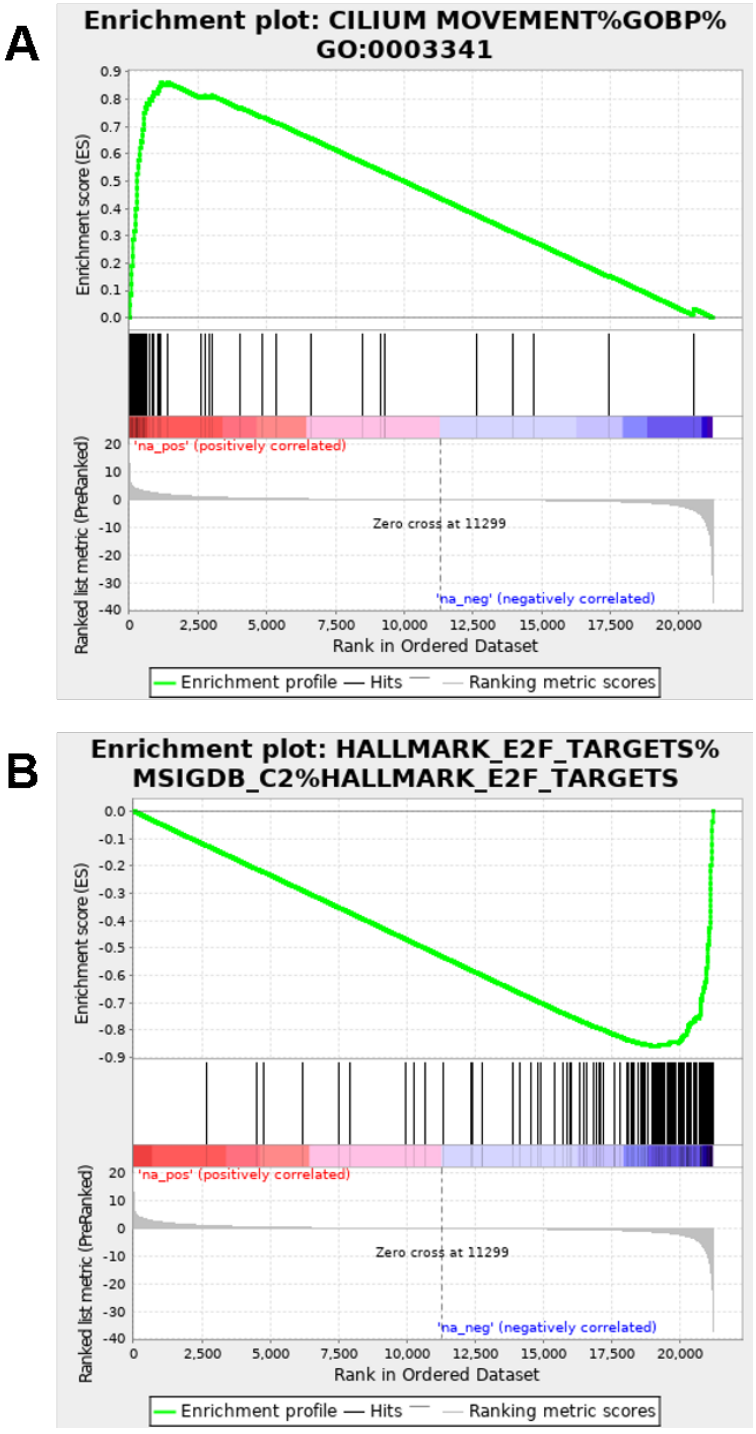

Figure 9: **Examples of GSEA enrichment plots**
(**A**) Enrichment plot for cilium movement, the first gene set with highest NES in upregulated genes following E2f1/3 knockout.
(**B**) Enrichment plot for hallmark E2f targets, the first gene set with lowest NES in downregulated genes following E2f1/3 knockout.

3.3 E2f Binding of Differentially Expressed Genes

In order to start parsing through differentially expressed transcripts in adult EKO NSCs, data was compared to previous transcriptional binding data. The study in question assessed E2f3 and E2f4 binding in neural precursor cells, eventually uncovering "hundreds of transcriptionally active E2f-bound promoters corresponding to genes that control cell fate processes, including key transcriptional regulators" (Julian et al., 2016). We took a similar approach, overlapping ChIP-on-CHIP data with the lists of differentially expressed genes following E2f1/3 knockdown in sorted cells (Figure 10). Note that while binding data is only available for E2f3 and E2f4 in these precursor cells, there is often very high overlap in E2f1 and E2f4 binding sites given their opposing effects on transcription through the DREAM/MuvB and RB-E2f complexes (Fischer & Müller, 2017a). The two data sets highlighted various groups of genes bound by E2fs: 738 and 779 differentially expressed genes following E2f1/3 knockout and also bound by either E2f3 or E2f4. Further comparison of these lists yielded a group of 407 differentially expressed genes that were bound by both transcription factors. These comparisons are presented in an alternate manner in Table 3, where directionality of this pool of RNAseq transcripts is taken into account. Presented in this way, it becomes clear that E2f binding is especially relevant in the group of 2,101 downregulated genes: roughly 25% of these are putative targets of either E2f3 and/or E2f4 in neural precursor cells. The integration of transcription factor binding data further nuances global transcriptional changes reviewed so far. The potential for E2f-mediated transcriptional control in adult NSCs for individual or large groups of genes lies at the heart of the following sections.

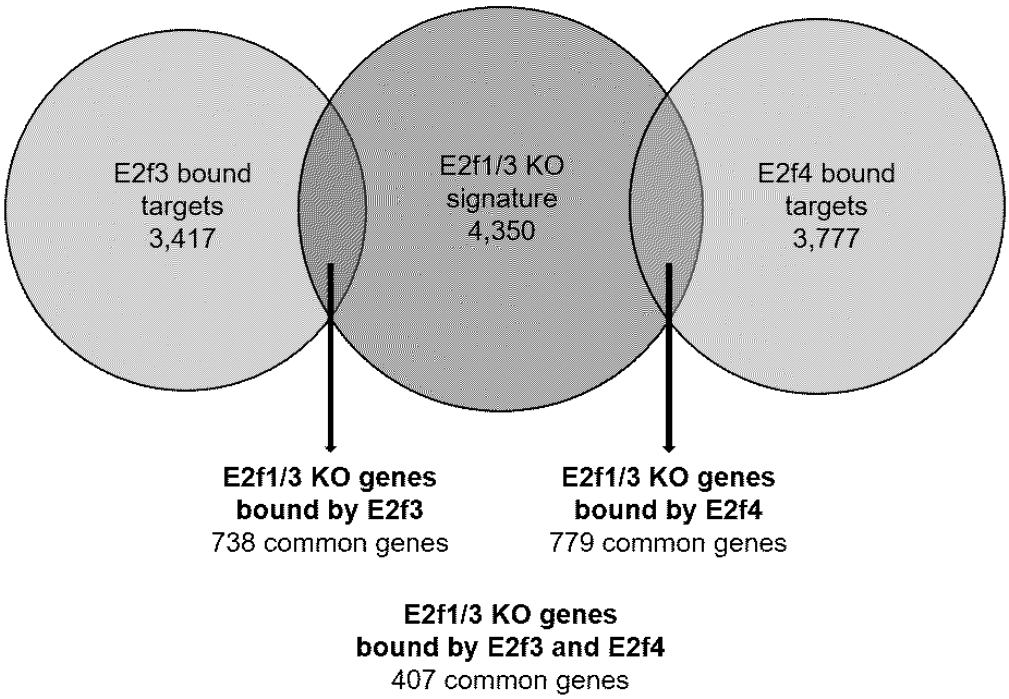

Figure 10: **A proportion of differentially expressed genes in adult NSCs lacking E2f1/3 are direct E2f targets**
Chart illustrating the overlap between differentially expressed genes in E2f1/3 knockout adult NSCs and E2f3 or E2f4 direct targets from embryonic NSCs. Binding data from ChIP-chip experiments previously done in this lab by L. Julian (Julian et al., 2016).

Table 3: **A proportion of differentially expressed genes in E2f1/3 knockout adult NSCs are direct E2f targets**

	Total genes	**Bound by E2f3 [% of total]**	**Bound by E2f4 [% of total]**
Upregulated with E2f1/3 KO	2,249	214 [9.5%]	217 [9.6%]
Downregulated with E2f1/3 KO	2,101	514 [24.5%]	562 [26.7%]

E2f3 and E2f4 binding and directionality of gene expression in E2f1/3 knockout RNAseq dataset.

3.4 Regulators of Neurogenesis Affected by Loss of E2f1/3

Based on results presented in Chapter 3.2, a more in-depth understanding of general transcriptome fluctuations in adult NSCs was needed in order to truly understand the ramifications of E2f1/3 knockout beyond cell cycle and E2f target disruptions. As such, ranked GO lists based on both upregulated and downregulated genes were used to create an enrichment map of the data as a whole (Figure 11). Once again, similar clusters preferentially emerge when plotting the full lists of modified genes together: cell cycle and mitosis, DNA processing, cilium and microtubules, etc. However, a novel category also emerges in this holistic view: neurogenesis and forebrain development. Generally, this echoes the preliminary *in vivo* characterizations presented in Chapter 3.1, where the model's neurogenic and NSC pool deficits were evident.

In a similar manner, full transcriptional data was used to identify the affected molecular pathways in adult NSCs following activator E2f knockout using KEGG Automatic Annotation Server (KAAS) (Table 4). Beyond cell cycle perturbations, pathways impacting cellular senescence and stem cell pluripotency are now being identified. Similarly, many signaling pathways crucial to stem cell identity are fate decisions are also in flux. In particular, our understanding of the neurogenesis network of processes from the previous figure is further dissected: neurodegeneration, neural development, and neurotransmission span the full developmental changes potentially encountered when going from NSC to terminally differentiated neuron in the adult brain. The association between neurodegenerative or disease states and neurogenic defects is an easy one to make (Sugaya & Vaidya, 2018; Toda et al., 2019); yet this also further underlines the great necessity to understand how these basic NSCs function before they are activated by disease or damage. Moreover, changes within the actin cytoskeleton category of neural

development may now be linked with the previously observed upregulation of cilia-related genes. General shifts in metabolism and neurotransmission might indicate changes in any of the cell populations targeted with the IHC analyses. Taken together, data from the enrichment map and pathway analyses reflect a distinct shift in our interpretation of the observed dynamics within the transcriptome: the removal of E2f1/3 from adult NSCs seems to have independent ramifications on both cell cycling but also during the general process of differentiation. While the specific cause of these neurogenic defects cannot yet be identified, changes within the transcriptome strikingly echo the initial *in vivo* characterizations.

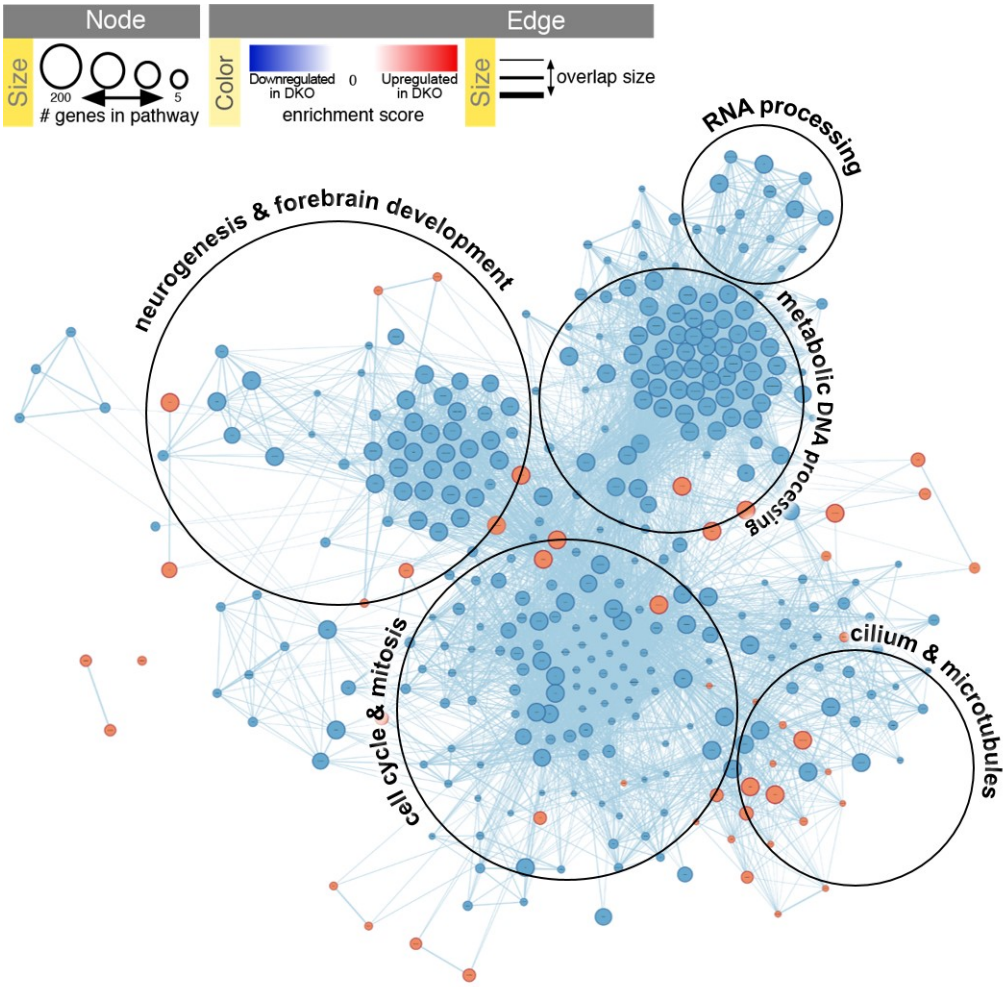

Figure 11: **Enrichment map of E2f1/3 knockout signature**
Enrichment map labelling most relevant clusters in previous combined upregulated and downregulated GO analyses. Blue nodes are downregulated and orange nodes are upregulated in E2f1/3 Knockout (KO) RNAseq dataset. Size of nodes reflects number of genes in a particular cluster.

Table 4: **Transcriptome changes following E2f1/3 knockout affect pathways regulating metabolism, cell cycle, and various aspects of neurogenesis or neural function**

Metabolism	**Neurodegeneration**
Metabolic pathways	Alzheimer's disease
Biosynthesis of 2° metabolites	Huntington's disease
	Parkinson's disease
Cell Cycle	
Pathways in cancer	**Neural Development**
Cell cycle	Regulation of actin cytoskeleton
Cellular senescence	Axon guidance
Signaling pathways regulating pluripotency of stem cells	Neuroactive ligand-receptor interaction
p53 signaling	
	Neurotransmission
Signaling Pathways	Dopaminergic synapse
PI3K-Akt signaling pathway	Cholinergic synapse
MAPK signaling pathway	Glutamatergic synapse
Hippo signaling pathway	Serotonergic synapse
Calcium signaling pathway	Synaptic vesicle cycle
Wnt signaling pathway	

Functional pathways modified by differentially expressed genes after knockdown were determined using the KAAS (Moriya et al., 2007). Pathways were sorted by number of hits and manually clustered into 6 overarching groups. These represent only the most significant fraction of perturbed pathways. Others were omitted due to similarities or redundancies with current groupings (e.g. MicroRNAs in cancer or Breast cancer relate to Pathways in cancer and Cell cycle, given the loss of cell cycle activators) or low number of hits within the pathway.

3.5 Enrichment of a Quiescence Signature Following Loss of E2f1/3

So far, Chapters 3.1 to 3.4 reflect work done following my first aim, that is to gain an overarching understanding of the effects of E2f loss in adult NSC at the cellular and molecular levels. The remaining sections' analyses were carried out to evaluate whether or not these changes to neurogenesis involved any shifts in NSC quiescence and activation. In order to evaluate this, E2f1/3 EKO

transcriptional data was compared to the list of markers for qNSCs and aNSCs generated by Codega et al. An overview of the comparison may be seen in Figure 12. When looking at differentially expressed genes that also figure on the generated signatures for quiescent or activated NSCs, a convincing trend emerges. When activator E2fs are absent in adult NSCs, the cells' transcriptomes are strikingly enriched for the established signature for quiescence (with activation genes conversely decreasing). This seems to identify a possible role for E2f1/3 in NSC exit from quiescence, independent from any downstream mechanisms involved in differentiation. Given the often antagonistic mechanisms by which the Rb/E2f network functions, it seemed relevant to make this comparison with an contrasting model. Using an established pocket protein triple knockout model (Vandenbosch et al., 2016) and the equivalent adult RNAseq dataset, the opposite effects for quiescence and activation signatures were observed (Figure 13). Loss of control by Rb means activator E2f expression is no longer curtailed, with the cells' transcriptome shifting towards a signature closest to NSC activation and a decrease in qNSC gene expression. The shift in expression of these key qNSC and aNSC signature genes was confirmed by RT-qPCR on independent samples (Figure 14). The expression shifts in these genes, which come from the quiescence and activation signatures from Codega et al., are comparable to transcript changes from original RNAseq data (Table 5).

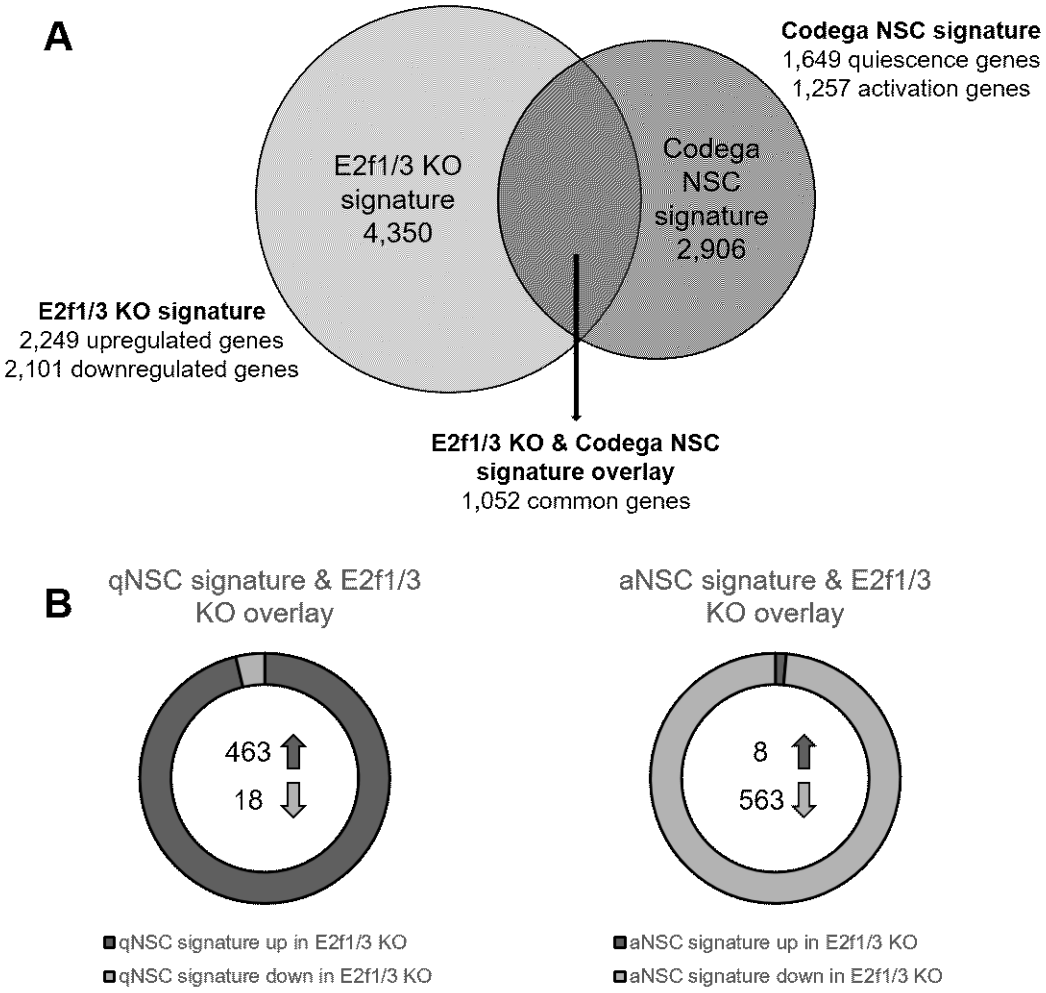

Figure 12: **Enhanced quiescence and reduced activation signatures in adult NSCs after E2f1/3 loss**
(**A**) Chart illustrating the overlay of gene lists for differentially expressed transcripts in both E2f1/3 knockouts and NSC quiescence and activation marker signatures. Quiescence and activation signatures are from a previously published characterization (Codega et al., 2014).
(**B**) From the overlay generated in A, quiescence and activation markers were grouped. Quiescence-associated markers were upregulated following E2f1/3 knockout in adult NSCs. Similarly, activation-associated markers were downregulated following E2f1/3 knockout in adult NSCs.

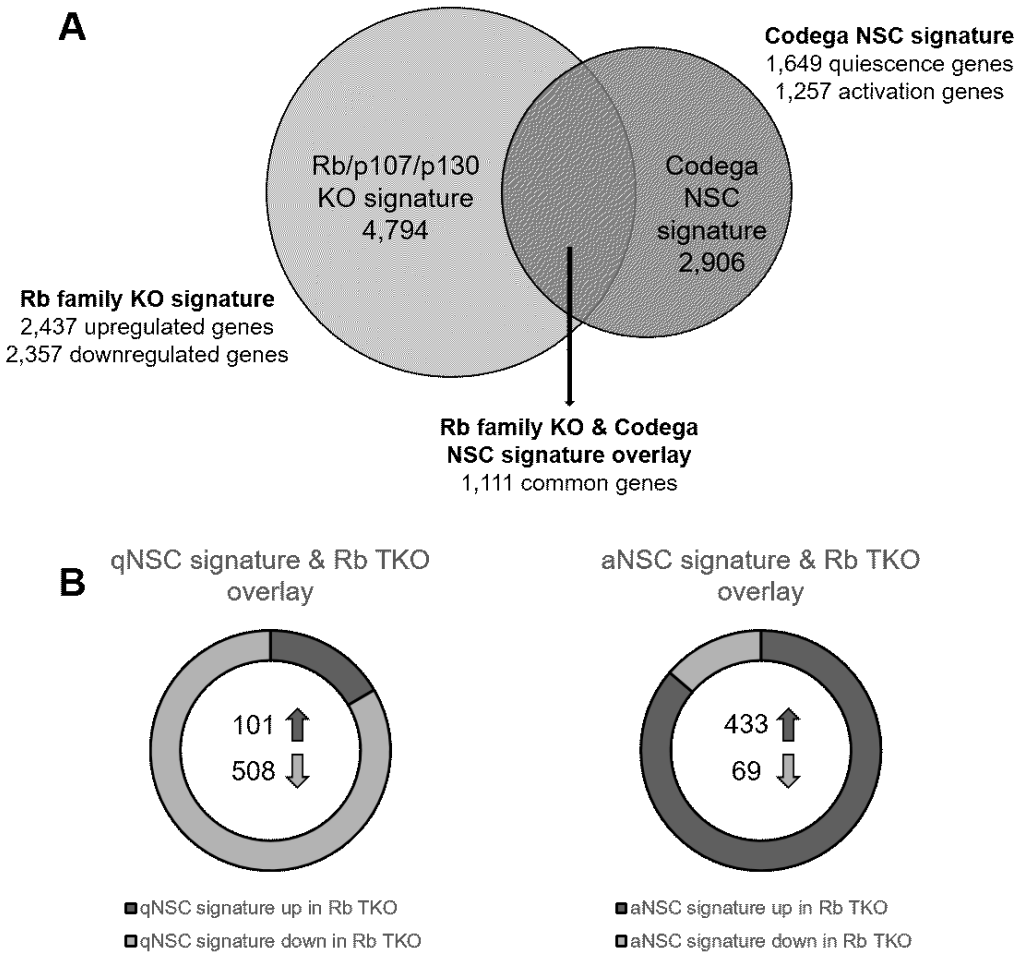

Figure 13: **Enhanced quiescence and reduced activation signatures in adult NSCs after Rb/p107/p130 loss**
(**A**) Chart illustrating the overlay of gene lists for differentially expressed transcripts in both Rb family knockouts and NSC quiescence and activation marker signatures. Quiescence and activation signatures are from a previously published characterization (Codega et al., 2014).
Rb knockout data from B. Fong.
(**B**) From the overlay generated in A, quiescence and activation markers were grouped. Quiescence-associated markers were upregulated following Rb family knockout in adult NSCs. Similarly, activation-associated markers were downregulated following Rb family knockout in adult NSCs.

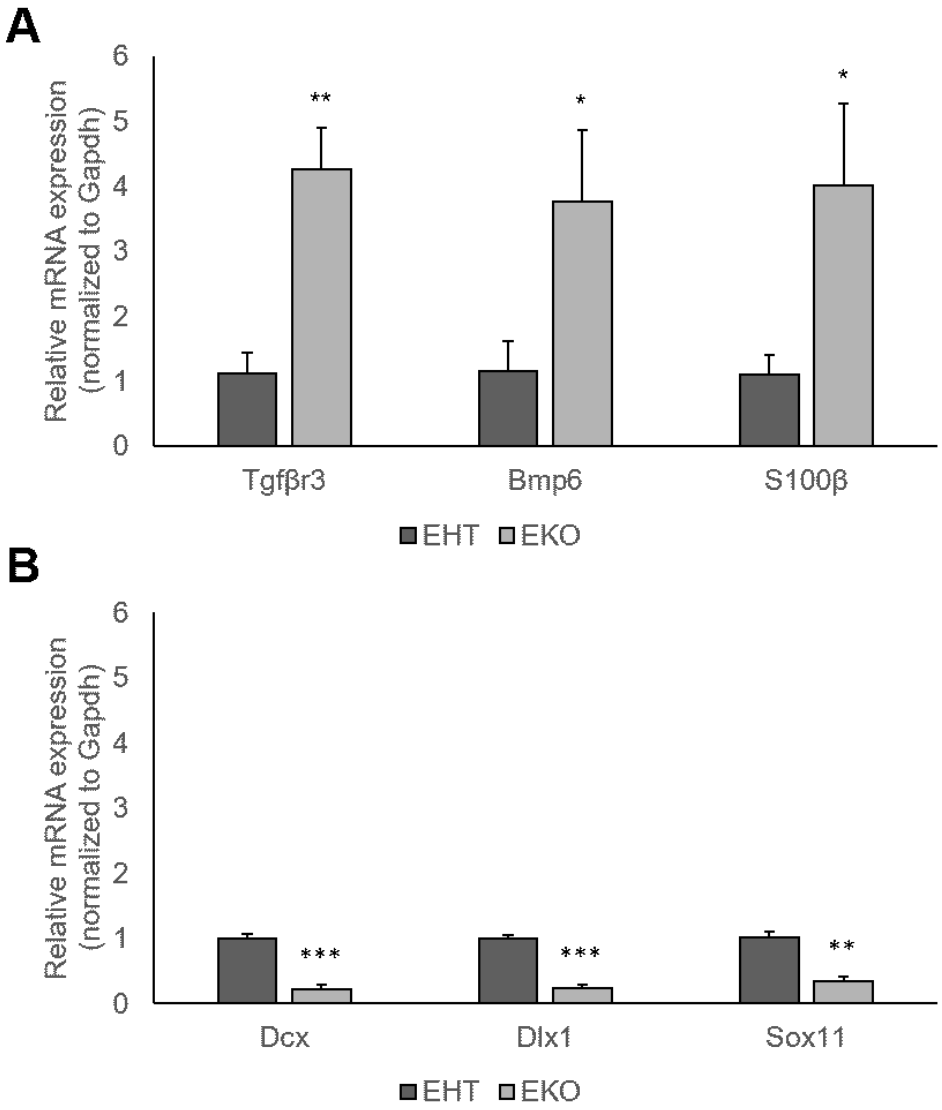

Figure 14: **Validation of quiescence and activation expression signatures in adult NSCs**
(**A**) RT-qPCR from RNA extracted from adult NSCs. Targets assessed represent previously identified markers for quiescence. n = 3, * $p < 0.05$, ** $p < 0.01$, one-tailed T-Test. Error bars show standard error.
(**B**) RT-qPCR from RNA extracted from adult NSCs. Targets assessed represent previously identified markers for activation. n = 3, ** $p < 0.01$, *** $p < 0.001$, one-tailed T-Test. Error bars show standard error.

Table 5: **Validation of quiescence and activation expression signatures in adult NSCs**

Gene	Log2 fold change	p-adj
Bmp6	1.914	1.55^{-10}
Dcx	-3.189	4.39^{-27}
Dlx1	-2.988	2.58^{-18}
S100β	1.458	2.92^{-3}
Sox11	-3.274	3.27^{-34}
Tgfβr3	1.826	4.03^{-4}

Table showing quiescence and activation marker transcription level fold-change and statistical significance from E2f1/3 knockout RNAseq.

The comparison of the E2f1/3 knockout signature genes with the qNSC/aNSC signature generated a list of 1,052 differentially expressed genes that are relevant in adult NSCs. Similarly to initial overviews, this list of enriched genes was once again analysed with g:Profiler to identify enriched ontologies related to biological processes (Table 6). Following E2f knockdown, there is upregulation of a wider variety of processes: of note, there are a few key metabolic changes, cell communication and signaling regulation, and several developmental processes. As always, downregulation of cell cycle processes remains consistent. Given the appropriate comparisons with established molecular signatures, a distinct expression profile that was otherwise masked by cell cycle effects may now emerge. These data seem to indicate the potential role for activator E2fs specifically in regulatory pathways relating to adult NSC exit from quiescence or transition to an activated state.

In order to specify the role of activator E2fs within the full range of changes during differentiation (and not just the qNSC/aNSC transition), RNAseq transcript expression was compared to key developmental markers as described by Basak et al (Figure 15). Using single-cell RNA sequencing, the team managed to generate a highly ordered list of sequential cell markers. When considering the full continuum of differentiation from qNSC to neuron, E2f1/3 EKO data

shows upregulation of quiescent and primed quiescent NSC markers, with the transition to aNSC markers coinciding with reduced transcript expression for the remaining genes of the panel. Whether comparing signatures for NSCs or spanning the whole of the neural lineage, the conclusions remain strikingly consistent: when absent, the activator E2fs are responsible for a transcriptional shift that roughly equates to enhanced quiescence and a decrease in NSC activation. This effect is supported through extensive direct but also indirect (i.e. pocket protein-related) validations. These changes correspond to a decrease in overall neurogenesis and NSC activation and an increase in cell cycle exit *in vivo*. A portion of these effects may even be regulated by direct binding of E2f1 or E2f3 to specific gene loci. The identification of a potential adult regulator of neurogenesis based on these analyses is described in this final section.

Table 6: **Individual gene ontology analysis of NSC quiescence and activation markers differentially expressed following E2f1/3 knockout**

Upregulated in EKO		**Downregulated in EKO**	
Gene	**p-value**	**Gene**	**p-value**
Regulation of localization	1.02^{-13}	Cell cycle	2.37^{-65}
Lipid metabolic process	7.46^{-12}	Cell cycle process	1.40^{-63}
Negative regulation of cellular process	2.32^{-11}	Mitotic cell cycle process	1.47^{-61}
Regulation of multicellular organismal process	2.63^{-11}	Mitotic cell cycle	4.28^{-59}
Regulation of cell communication	3.39^{-11}	Chromosome organization	5.60^{-52}
Regulation of signaling	4.95^{-11}	Cell division	3.82^{-44}
Negative regulation of biological process	9.14^{-11}	Chromosome segregation	2.11^{-38}
System development	1.94^{-10}	DNA metabolic process	3.24^{-38}
Anatomical structure development	2.11^{-10}	Organelle organization	7.94^{-38}
Multicellular organism development	2.49^{-10}	Nucleic acid metabolic process	1.89^{-37}

Gene ontology analysis for biological processes run with gProfiler on differentially expressed gene transcripts in E2f1/3 knockout RNAseq that coincided with an established quiescence or activation signature. First 10 classifications shown for each set of genes.

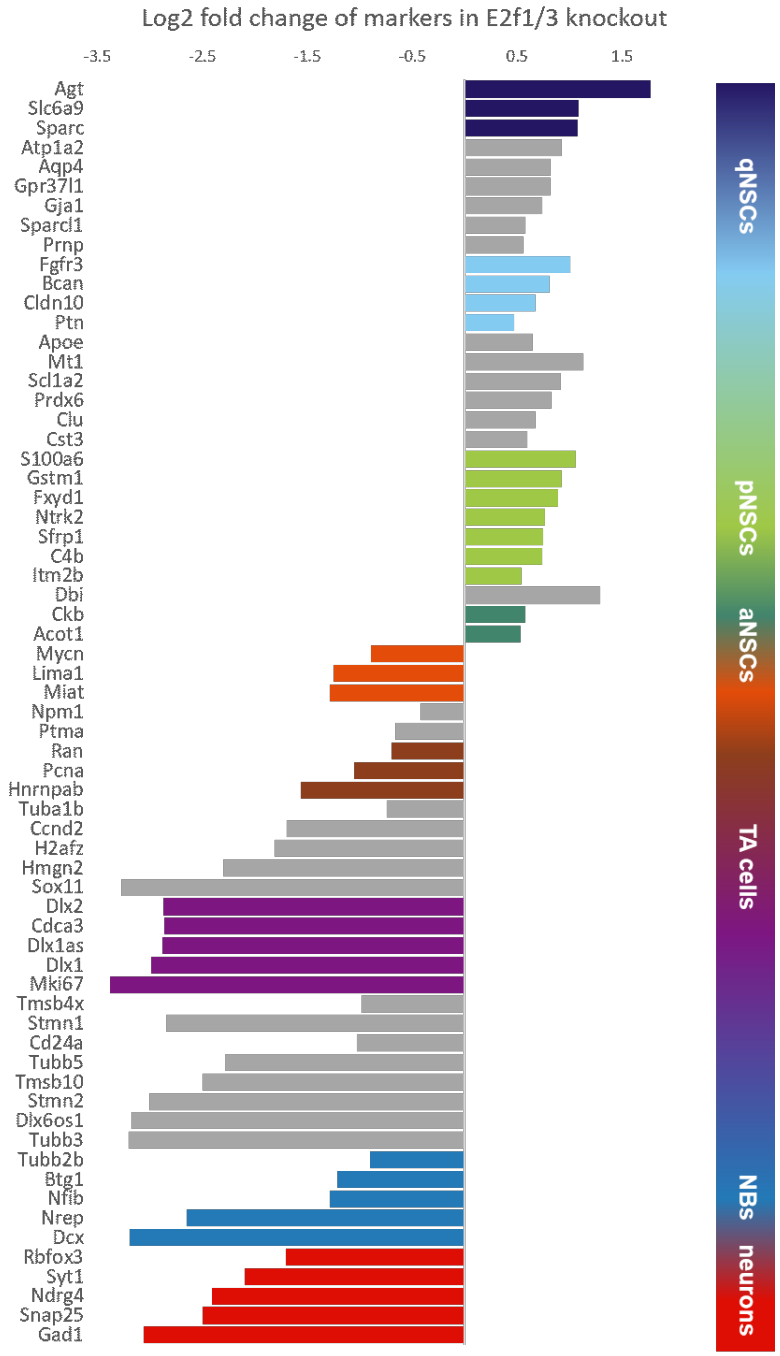

Figure 15: **Transcript expression of adult E2f1/3 knockout NSCs corresponding to identity markers from quiescent NSCs to differentiated neurons**

Graph illustrating the log2 fold change of identified markers (Basak et al., 2018) from RNAseq following E2f1/3 knockout. Generally, gene transcripts associated with quiescence are upregulated until the primed/activated NSC transition, where transcription decreases for genes associated with activated NSCs through to terminally differentiated neurons. Genes with grey bars represent markers for two or more numbered clusters (broader spectrum markers). Original published t-SNE map can be found in Figure 19.

3.6 Akna: A Potential E2f Target Affecting Neurogenesis in the Adult

A relatively recent publication described the crucial role of AT-Hook Transcription Factor (Akna), an obscure transcription factor that coordinates the centrosomal microtubule network, during developmental neurogenesis of the SVZ (Camargo Ortega et al., 2019). Based on our RNAseq data set, Akna levels decrease significantly following E2f1/3 knockout (Table 7). This was confirmed by RT-qPCR on independent samples (Figure 16). Within the context of this E2f1/3 adult knockout model, their loss also coincides with decreased Akna expression. Using previously referenced embryonic ChIP-on-CHIP data in mutant E2f3 or E2f4 cells, two Akna binding sites upstream of the gene's Transcription Start Site (TSS) were identified (Figure 17). Given its binding during neural development and its reduced transcription following activator E2f knockout, E2f binding at either of the two identified chromatin regions was assessed with ChIP on adult neurospheres. Using Cell Division Control Protein 6 Homolog (Cdc6) and Voltage-Dependent Calcium Channel Gamma-1 Subunit (Cacng1) as positive or negative controls respectively, it was demonstrated that there was significant binding of E2f1 and E2f4 at the furthest site from the TSS compared to background enrichment. Taken together, these data start to make a case for the investigation of E2f-mediated regulation of Akna within NSCs as having potential effects on adult neurogenesis.

Table 7: **Reduction in Akna mRNA levels following E2f1/3 knockout**

Gene	Log2 fold change	p-adj
Akna	-0.798	0.031335

Table showing transcription level fold-change and statistical significance from E2f1/3 knockout RNAseq.

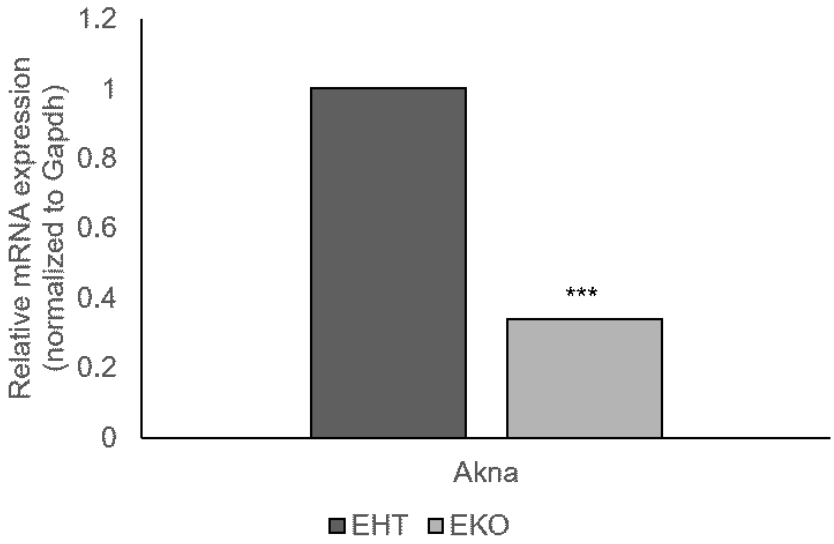

Figure 16: **Reduction in Akna mRNA levels following E2f1/3 knockout** RT-qPCR from RNA extracted from adult NSC. Tissue dissected 10 DPI from the SVZ, followed by YFP^+ cell isolation with FACS. n = 3, *** $p < 0.001$, one-tailed T-Test. Error bars show standard deviation.

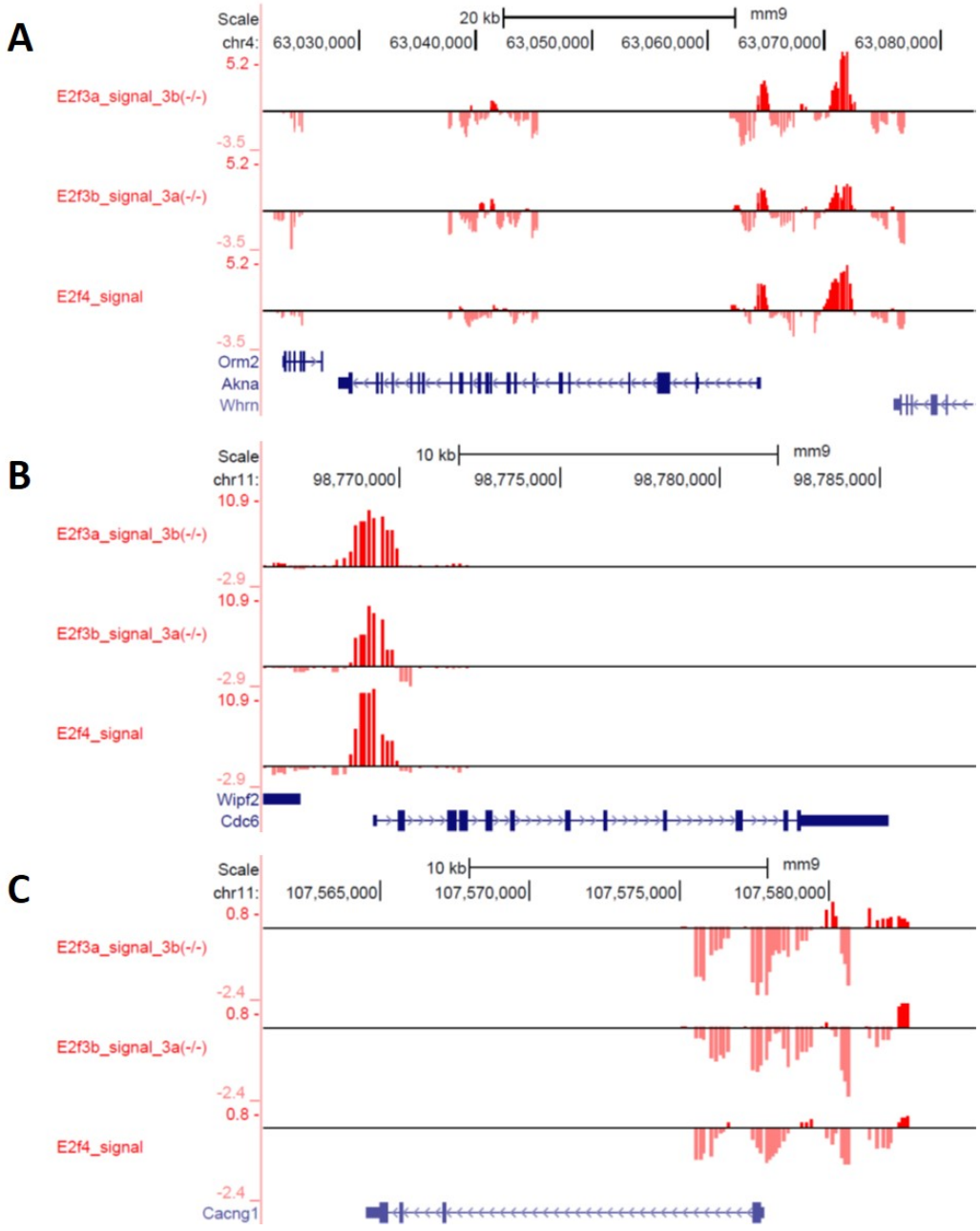

Figure 17: **E2f binding at the Akna promoter during embryonic development**
(**A**) Genomic binding profiles for E2f3a, b, and E2f4 in E14.5 neurospheres upstream of the Akna promoter. All 3 E2fs were significantly enriched at these sites. Data from ChIP-chip experiments previously done in this lab by L. Julian (Julian et al., 2016).
(**B**) Genomic binding profiles for E2f3a, b, and E2f4 at the Cdc6 TSS. Cdc6 is a known E2f target. All 3 E2fs were significantly enriched at these sites.
(**C**) Genomic binding profiles for E2f3a, b, and E2f4 at the Cacng1 locus. This gene serves as a control region, being mostly expressed in muscle. No significant enrichment at this site.

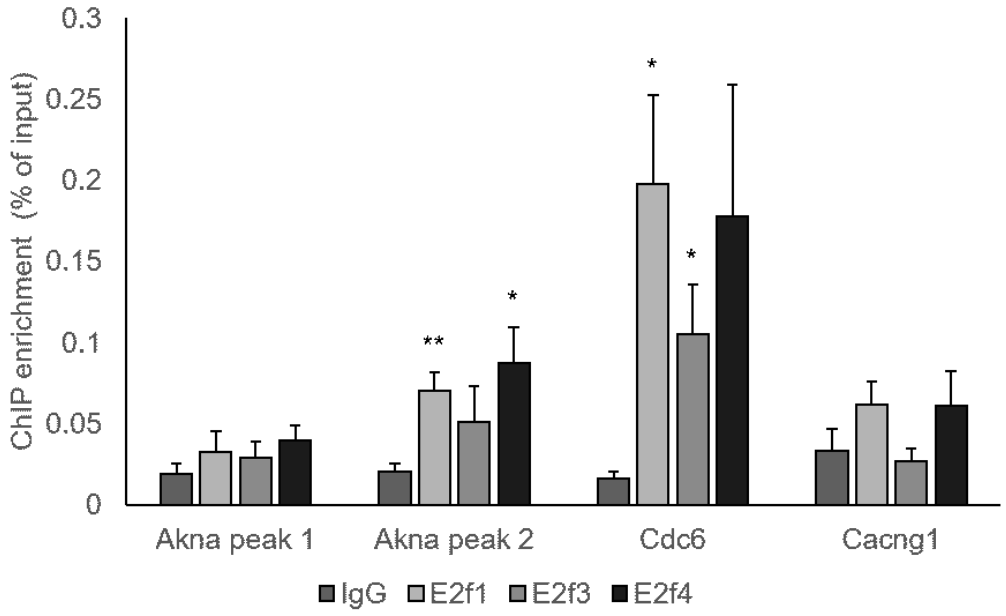

Figure 18: **E2f binding at the Akna promoter in adult NSCs**
ChIP RT-qPCR on chromatin from Wild Type (WT) adult NSCs. n = 8 for IgG and E2f3, n = 5 for E2f1 and E2f4, * $p < 0.05$, ** $p < 0.01$, two-tailed two sample unequal variance T-Test. Error bars shown as standard error.

3.7 Summary of Results

In sum, this thesis offers substantial proof for an expanded role of the activators E2f1 and E2f3 during adult neurogenesis, specifically in the regulation of quiescence and activation of NSCs. The inducible transgenic model allows for characterization of the neurogenic niches (in this case, with particular attention on the SVZ) and the overall decrease in neurogenesis and pro-neural activation following deletion of E2f1 and E2f3. A combination of *in vivo* IHC and bioinformatic approaches painted a cohesive picture where these transcription factors are behind the large scale transcriptome shifts that effectively modulate the balance between quiescent or active NSC profiles. In particular, a large proportion of downregulated genes are directly bound by E2fs. Beyond the typical cell cycle effects, loss of E2fs impacts entire pathways related to metabolism, cellular signaling, neural development, and

neurotransmission. Furthermore, complimentary models knocking down either the activator E2fs or pocket proteins directly link E2f1 and E2f3 expression with enhanced NSC activation. These effects are predominantly focused on NSC quiescence and activation when considering the full developmental transformation of stem cell to postmitotic neuron.

This combination of methods builds an effective pipeline for prospectively identifying novel neurogenic candidates in adult NSCs and could help understand the mechanistic roles of E2f1 and E2f3 in overall neurogenesis and exit from quiescence. Preliminary data suggests that Akna may be one of these targets worth pursuing. Taken together, these findings carve out a role for activator E2fs that is independent from cell cycle progression. Within the context of adult neurogenesis, E2f1 and E2f3 are required for exit from quiescence and activation of NSCs ahead of differentiation.

Chapter 4

Discussion

4.1 The Role of Activator E2fs in the SVZ

Following the generation of the E2f1/3 knockout mouse model, initial characterizations focused on understanding the extent of the neurogenic defects caused by the removal of activator E2fs. Prior to this, models for individual activator E2f knockdown mostly demonstrated a modest effect on neurogenesis and cell proliferation (Wu et al., 2001; Cooper-Kuhn et al., 2002; McClellan et al., 2007). The rationale for this project finds its roots in the initial characterization of this E2f1/3 model, where defects in neurogenesis impacted all SVZ and SGZ metrics by almost one order of magnitude (Yakubovich, 2019). While transcript abrogation for E2f3 specifically remained inexplicably variable throughout multiple independent isolations (Figure 5), the significant impact on neurogenic capability was deemed adequate for study. It is known that, given their auto-regulatory nature, E2f deletion must often be performed across multiple members to achieve sufficient disruption (Wu et al., 2001).

The IHC stain with CD133, Gfap, and Egfr based on work by Codega et al. confirms most directly the effect of activator E2f knockout on the balance of quiescent and active NSCs in the SVZ. As a reminder, the two initial aims chosen to guide this study pertained first to the characterization of the loss of

activator E2fs, then explored the requirement for these for exit from quiescence in adult NSCs: these *in vivo* data show this most plainly (Figures 6 and 7). By using a panel of markers to pick out both populations of NSCs, it is possible to compare their relative dynamics shift. Markers for these assays must be chosen judiciously in order to isolate the required populations from the neural lineage (Kuhn et al., 2016; Zhang & Jiao, 2015). Similarly, a small caveat that must be kept in mind throughout this analysis is that nestin expression has been shown to be widely variable depending on the phase of the cell cycle (Sunabori et al., 2008). This has potential ramifications when using a nestin-driven Cre model, as the targeted cell populations are not guaranteed to stem from NSCs that have recombined following tamoxifen administration (for instance, in the direct comparison between Figure 6 and 7). Nevertheless, the proportion of active NSCs within the SVZ reduces significantly and forms the first piece in building a case for the role of activator E2fs prior even to commitment and transition to a progenitor state. For this and subsequent sections, the subtle shifts in cell populations isolated based on various markers must be kept in mind. However, a global reduction in aNSCs over time is the undeniable starting point in appreciating cell population shifts.

Given the slight differences in markers used (Figure 7), quantifications based on Sox2 expression take into account a slightly wider proportion of stem-like NSCs. Here, widespread p16 expression is roughly equated to cell cycle exit. While this may be the case, there are various reasons for which cell cycle machinery may be driving them to exit (one of these being quiescence) (Rayess et al., 2012; Molofsky et al., 2006). Ideally, a more complete case would be required to ascertain true cellular quiescence distinct from, for instance, senescence. However, these explorations go beyond the scope of this thesis but would remain relevant within a neurogenic context (He et al., 2013). Taken together, however, the defects represent significant losses of neurogenic potential at a very young age (either 4 or up to 8 weeks postnatally). As the

short-term effects of an E2f1/3 knockout in the adult brain point very strongly to a lack of NSC activation and an increase in withdrawal from cell cycle, this mutant line could reveal relevant long-term insights as a model for accelerated ageing via niche exhaustion (Kalamakis et al., 2019; Navarro Negredo et al., 2020).

4.2 Modulation of Quiescence and Activation by the Activator E2fs

Much of this study is exploratory in nature (Aim 1), as successive analyses and comparisons to other datasets were required to further refine our view of the transcriptional impacts following loss of the activator E2fs in adult NSCs. Gene ontology analyses on downregulated genes offer perhaps the most intuitive global shift: the generalized dampening of the cell cycle and mitotic processes, including hallmark E2f and checkpoint targets (Tables 1 and 2). Given their ability to modulate transcription, the comparison to ChIP-chip data from adult NSCs was a logical first step (Figure 10) (Julian et al., 2016). Likely due to the broad differences in the cell populations sampled (freshly sorted cells from the SVZ, compared to neurospheres passaged in culture), the overlap between these signatures is strikingly slim for both E2f3- and E2f4-bound targets. Though a wide breadth of E2f target genes have been studied since their discovery (McClellan & Slack, 2007; Giacinti & Giordano, 2006), it is interesting that significant proportions (up to roughly a quarter, and especially for downregulated targets) of differentially expressed genes from the RNAseq dataset are putative E2f targets (Table 3).

Visualization of expression data via an enrichment map (Figure 11) offers our first description of the activator E2fs' roles beyond mitotic coordination, during neurogenesis and forebrain development. Once again, this reiterates solidly established roles for E2f1 and E2f3, through direct or indirect means, in

fate determination and differentiation throughout the neural lineage (Julian et al., 2016; Ohnuma & Harris, 2003; Urbach & Witte, 2019). The functional annotation of differentially expressed transcripts through KAAS across broad pathways begins to elucidate changes brought upon by loss of E2f1/3 in these adult NSCs (some of which are introduced in sections 1.2.2 and 1.2.3). Generally, these data directly echo previously characterized contributions by the E2fs towards metabolism (Kent & Leone, 2019; Nicolay & Dyson, 2013; Fajas et al., 2002), neurodegeneration (Nguyen et al., 2002; Höglinger et al., 2007; Tuerxun et al., 2021), neural development (as previously mentioned), and neurotransmission (Reynolds et al., 2014; Ting et al., 2014). Though this is by no means an exhaustive list, it is relevant to note the generalized triangulation between the E2fs' pleiotropic roles, the context of cellular stemness, and the interaction through these common pathways as they converge to shape the cellular milieu in any given moment (Hughes & Brady, 2005; Chaussepied & Ginsberg, 2004; Xiao & Dong, 2021). Similarly, the breakdown of the cell cycle header to include both pluripotency networks, cellular senescence, and p53 signaling further nuances the description of the possible cellular states reached by adult NSCs lacking activator E2fs (Sherr & McCormick, 2002; Harris & Levine, 2005). Taken together, this analysis of disregulated pathways in the mouse model further highlights separate (albeit interrelated) roles the activator E2fs play in either cell cycle progression or function/identity in the neural lineage and is fully supported by our current knowledge across all of these topics. So far, the characterizations made throughout Aim 1 are also consistent with expected effects following a loss of E2f1 and E2f3 in adult cells.

However, the crux of this thesis (Aim 2) must carve out a distinct role for the activator E2fs ahead of differentiation, within the dynamic balance of qNSCs and aNSCs. Figure 12 offers a direct comparison with a known signature of quiescent and activated states in subventricular NSCs. Once again, discrepancies in overlapping genes could stem in part from minor differences

between isolation methods and models used in these studies. However, general trends are clear on both sides: common genes belonging to the quiescence signature are almost all upregulated (96%). The opposite is true for the downregulation of the activation signature (98%). Generally, the opposite effect manifests when using an equivalent, contrasting model without pocket proteins (Figure 13). The shifts in transcriptional profiles based on expression data from this model are slightly less pronounced, likely due to roles unique to pocket proteins divorced from the Rb/E2f axis (Dick & Rubin, 2013). At this point, it should be noted that transcriptome-level analyses of any cell sample (through RNAseq, RT-qPCR, etc.) offers an imperfect snapshot as it cannot account for various downstream processes such as translation, modulation of transcript stability, or splicing events (Stark et al., 2019). However, the transcriptional shifts caused by loss of E2f1/3 in NSCs solidly demonstrate individual posturing towards a state of quiescence and away from a one of activation. This framework of a double enhancement/depression effect caused by distinct transcriptional mechanisms is echoed later in this Discussion.

Finally, secondary analyses of gene ontologies based on overlapping signatures with RNAseq data further confirm this global shift within the transcriptome (Table 6). Interestingly, the top biological processes identified for both up- and downregulated markers closely mirror established differences in molecular dynamics between qNSCs and aNSCs (Shin et al., 2015; Urbán et al., 2019). Working from established molecular signatures for quiescence and activation, NSCs from this model show strong, consistent shifts towards the former. Comparisons within a broader context once again recapitulate these transcriptional shifts across a panel of markers spanning qNSCs to postmitotic neurons (Figure 15). Again, we see an overexpression of quiescent (and in this secondary case, primed) markers with loss of activator E2fs as well as an opposite decrease in transcripts associated with activation through differentiation. This offers a final re-centering of the model on yet another

bank of markers generated based on an individual lineage. In sum, these data not only align with our current understanding of quiescence markers and cellular halmarks (Kim et al., 2017; Delgado et al., 2021; Urbán et al., 2019), but also novelly carve out an additional role for the activator E2fs specifically in the balance between quiescence and activation in adult NSCs. Conditions to fully validate this requirement in the future will be briefly discussed later.

4.3 Microtubule Dynamics

While investigating the role of the activator E2fs in adult NSCs from the SVZ, the contribution of microtubule dynamics and the cellular cytoskeleton have been brought into question at various independent points during this study (Figure 11 and Tables 1, 2, and 4). Generally, the interaction between microtubule dynamics and cell cycle progression is core to our basic understanding of mitosis (Andersen, 1999). Inexplicably, this biological process was consistently one of the most upregulated in all analyses following activator E2f knockout (even without quiescence/activation signature overlays) and remained a partial mystery, when viewed alongside the robust disruptions in the E2f network. In light of the previous conclusions, it is possible to view this overexpression as an indirect readout of the enhanced quiescent state adopted by the cells. Even more than an indicator of the cellular state, it has been hypothesized that these microtubules consist of molecular links that enable cellular division (Phua et al., 2019; Kim & Tsiokas, 2011).

A few relevant details stand out while observing the SVZ niche: the ciliated B1 astrocytes (resident NSCs) cluster in pinwheel formations on the walls of the lateral ventricles (Mirzadeh et al., 2008). While the cilia are an important part of the integration system for systemic or environmental cues (Ringers et al., 2020), they remain hallmarks of cells that are not actively cycling (Goto et al., 2017). As such, this upregulation of the microtubule dynamics is consistent within a

population of cells whose transcriptome is robustly shifted towards a quiescent signature in NSCs.

4.4 Akna as an E2f-Regulated Modulator of Neurogenesis

While it has been generally linked to cancer, immunity, and inflammation, Akna remains a mysterious candidate in most contexts (Savas & Liu, 2009; Landvik et al., 2009). As it relates to this study, the AT-hook transcription factor is mostly relevant for its key role during cortical delamination and early NSC fate (Camargo Ortega et al., 2019). Beyond the study of activator E2fs and neurogenesis, we have also built an effective pipeline to investigate and identify putative E2f targets as potential key players in neurogenesis. Though the data are rudimentary at the time, there is enough information (characterization study, transcriptional changes following loss of activator E2fs, binding data) to warrant further investigation. Interestingly, this protein is also located at the core of the centrosome, which neatly ties in the previous section's observations on cell cycle progression, the quiescent state, and the cell's microtubule network. What's more, the initial /glse2f characterizations demonstrated a specific role for /glse2f3 (but not the other E2fs) in the duplication of the genetic material and the centrosome structure during mitosis (Saavedra et al., 2003). However, the extent of these connections would need to be explored as its own distinct project.

4.5 The Rb/E2f Bistable Switch, Cell Cycle, and Quiescence

The data in this thesis make a solid case for the additional involvement of the activator E2fs during neurogenesis, specifically as it relates to the balance between quiescence and activation in adult NSCs. Being the basis for this study, the loss of E2f1 and E2f3 in adult cells nevertheless has far-reaching implications for perturbations within cell cycle pathways (Bertoli et al., 2013; Lim & Kaldis, 2013; Bretones et al., 2015). As we refine our understanding of the broad levers of cell cycle control, it is possible to adopt a perspective that encompasses the Rb-E2f axis as a whole. Expanding on their naturally antagonistic natures, this pathway has been proposed to work in a stochastic, bistable manner: at any given moment, cells integrate the E2f signals and actively support either an arrested or cycling state (Yao et al., 2008). Centered around the cell's R-point (which necessarily implies a decision to be made), the balance between these cellular states is described as being "high-threshold, low-maintenance" (Yao, 2014). These two states are actively maintained by positive feedback loops (Ferrell, 2002). Mathematical modeling has also supported available *in vitro* data (Kirunda et al., 2021; Pandey & Vinod, 2018).

Through the lens of stem cell activation, this non-cycling quiescent state stems directly from this Rb-E2f axis. What's more, the depth of cellular quiescence can be fine-tuned using these same levers (Kwon et al., 2017). This final example directly mimics conditions explored in this study, where findings all point towards an overwhelming shift in the transcriptome away from activation and towards quiescence. Though the model describes two stable cellular states, the depth of quiescence is flexible (and consequently, shifts the potency of mitogenic stimulus required to reactivate these cells) and is determined by the host of systemic, environmental, and molecular cues (Fujimaki et al., 2019; Fujimaki & Yao, 2020). Understanding how to

overcome this additional barrier of quiescence depth is crucial when elaborating therapies for cancer (Fujimaki & Yao, 2018) and, in this case, potentially rallying the local NSCs to counteract natural ageing, neurodegeneration, and brain injury (Ibrayeva et al., 2021; Rojas-Vázquez et al., 2021; Bobkova et al., 2020).

4.6 Future Directions

This study offers multiple viable avenues of investigation to further unravel the intricacies regulating NSC quiescence and activation. As previously mentioned, Akna is one promising candidate that might be novelly linked to the E2fs. In order to assess the viability of this project, similar characterizations would need to be completed within adult mice to understand the role of this transcription factor, to act as a bridge from the original embryonic study (Camargo Ortega et al., 2019). Similarly, any number of candidates could be investigated, working from expression data and novel discoveries from the field. For instance, ongoing work based on E2f and pocket protein knockout datasets aims at understanding this same requirement for the activator E2fs in the induction of proneural programs within cells (Bergsland et al., 2006).

Though this thesis focuses mainly on the SVZ, most experiments (with apropriate modifications) could be adapted for the SGZ to gain a more global understanding. Given the perspective adopted for this study, the next logical line of questioning would be to better understand the quiescent state adopted by these E2f1/3 knockout cells (and distinguish from possible senescence or complete cell cycle withdrawal) and offer a direct transcriptional mechanism through which these changes take place. The gold standard to evaluate senescence is achieved by assaying Senescence-Associated β-Galactosidase (SA-β-Gal) activity within the neurogenic niches and could be complemented by a more robust study of Cdk inhibitor markers (reminiscent of Figure

7) (Terzi et al., 2016; Chandler & Peters, 2013). While *in vitro* assays remain impossible with E2f1/3 knockout, *in vivo* experiments could be devised to further look into quiescence. Either through IHC panels (Codega et al., 2014) or birthdating studies, the cell populations (and quiescent/activated proportions) could be assayed over time, or following administration of an activating stimulus to prompt cell cycle re-entry (Ding et al., 2020). Morphological data following loss of activator E2fs could also be sought to see whether any global shifts in cells occur with these transcriptional changes (linking with section 4.3). Naturally, classical experiments breaking down cell cycle kinetics and rate of activation or turnover would provide the clearest understanding of this quiescent population, once identified (Zerjatke et al., 2017). Finally, with a tool used originally in studies looking at primitive NSCs through development (Furutachi et al., 2015) and crossing with the E2f1/3 knockout line, it would be possible to track and visualize NSCs through fluorescence. Though signal dilution and retention rates from the histones would be the limiting factors, many of these experiments could be reiterated within this framework to gain a deeper understanding of both maintenance of quiescence and the origins of NSCs as they persist through adulthood.

Chapter 5

Conclusion

These findings serve to further characterize the effects of activator E2f loss within adult NSCs. Building on the previously described neurogenic defects of the model (Yakubovich, 2019), *in vivo* data in the SVZ niche also shows decreased activation in the resident stem cell pool and increased cell cycle exit following loss of E2f1 and E2f3. Using a combination of relevant binding and expression data, loss of these factors correlates with distinct dampening of both cell cycle and pathways associated with neural development. Comparison of the resulting signatures with published datasets (Codega et al., 2014; Basak et al., 2018) reveals a distinct role for the E2fs in the maintenance of a quiescent cellular state and eventual activation towards differentiation. Preliminary data identify Akna as a putative E2f target related to neurogenesis. These data are in line with our current understanding of the Rb-E2f axis as it relates to cellular quiescence and cell cycle control. Understanding the role of the activator E2fs for exit from – and modulation of – quiescence is key to lifelong niche maintenance and successful NSC activation.

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Appendices

Figures

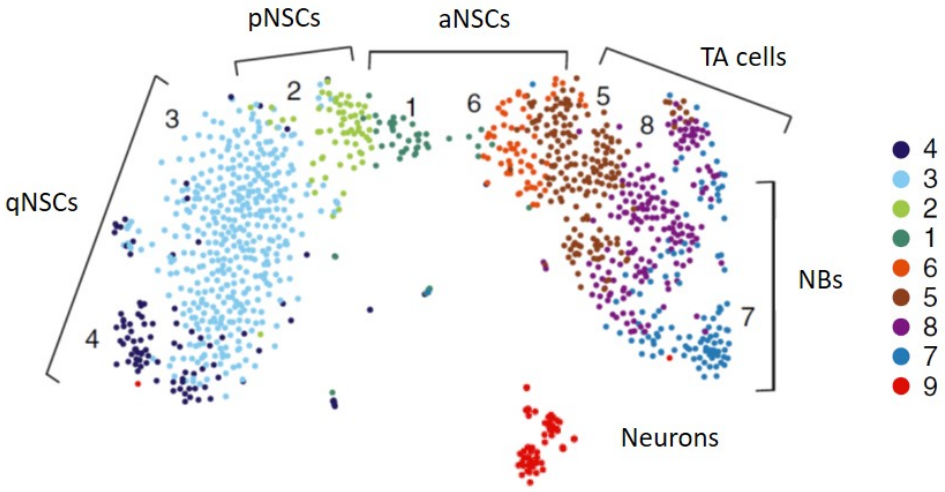

Figure 19: **Creation of clusters identifying cells from NSCs to neurons used in Figure 15**
t-SNE map (Basak et al., 2018) from single-cell transcriptome atlas of adult neurogenesis in the SVZ, showing individual clusters separated by identity. The group identified markers for quiescent, primed, and adult NSCs, transit amplifying cells, neuroblasts, and neurons.

Tables

Table 8: **Antibodies used for IHC and ChIP**

Antibody	Raised In	Manufacturer	Catalogue No.
Alexa Fluor 488 Secondary	Various	Jackson ImmunoResearch	Various
CD133	Rat	eBioscience	14-1331-82
Cyanine Cy3 Secondary	Various	Jackson ImmunoResearch	Various
Cyanine Cy5 Secondary	Various	Jackson ImmunoResearch	Various
E2f1	Rabbit	Santa Cruz	sc-193
E2f3	Rabbit	Abcam	ab50917
E2f4	Rabbit	Santa Cruz	sc-1082
Egfr	Rabbit	Abcam	ab52849
Gfap	Mouse	Chemicon Int.	MAB3402
IgG	Rabbit	Sigma-Aldrich	I 8140
p16	Mouse	Santa Cruz	sc-1661
Sox2	Goat	Santa Cruz	sc-17320

Animal source, supplier name, and catalogue numbers for each antibody have been included. Secondary antibodies against various species were used depending on the combination of primary antibodies used in the IHC stain.

Table 9: **Primer sequences used for genotyping and RT-qPCR**

Name	Sequence	Application
Akna F	ACCCCTCATCTTCAAGTCGC	RT-qPCR
Akna R	TAGCTCAGTGGCTTGTTCGG	RT-qPCR
Akna (peak 1) F	GAAGAGGAGCCTGCAGTTCC	ChIP
Akna (peak 1) R	ACACAGTGGGAGAGGCTTTG	ChIP
Akna (peak 2) F	AAGGTTGAGTTTCACGCCCC	ChIP
Akna (peak 2) R	CTTTGTCTGCCGCTCAACTC	ChIP
Bmp6 F	CAAGTCTTGCAGGAGCATCA	RT-qPCR
Bmp6 R	ATGTCAAATTCCAGCCAACC	RT-qPCR
Cacgn1 F	GACTTCTATTAATGCTTGTCACTAGGC	ChIP
Cacng1 R	GGGTATTTCTAGCCCTCAGATG	ChIP
Cdc6 F	TTCCTCCGAGGTCTCAAAAG	ChIP
Cdc6 R	CGGCAGCCAATAGGACAG	ChIP
Cre F	GAACCTGATGGACATGTTCAGG	Genotyping
Cre R	AGTGCGTTCGAACGCTAGAGCCTGT	Genotyping
Cre Control F	TTACGTCCATCGTGGACAGC	Genotyping
Cre Control R	TGGGCTGGGTGTTAGCCTTA	Genotyping
Dcx F	ACACCCTTGATGGAAAGCAG	RT-qPCR
Dcx R	TTCAGGACCACAAGCAATGA	RT-qPCR
Dlx1 F	GAACCGGAGGTTCCAACAA	RT-qPCR
Dlx1 R	TGACCTGCGTCTGTGTGAGT	RT-qPCR
E2f1 5'	GGATATGATTCTTGGACTTCTTGG	Genotyping
E2f1 3'	CTAAATCTGACCACCAAACGC	Genotyping
E2f1 PGK	CAAGTGCCAGCGGGGCTGCTAAAG	Genotyping
E2f1 F	CTGCAGCAACTGCAGGAGAG	RT-qPCR
E2f1 R	CTCCGAAAGCAGTTGCAGC	RT-qPCR
E2f3 A	GTGGCTGGAAGGGTGCCAAG	Genotyping
E2f3 B	TGAATCATGGACAGAGCCAGG	Genotyping
E2f3 C	GATTGATTCTGGGTTGTCAGG	Genotyping
E2f3 F	GGTCCTGGATCTGAACAAGGC	RT-qPCR
E2f3 R	CCTTCCAGCACGTTGGTGAT	RT-qPCR
Gapdh F	GGTGAAGGTCGGTGTGAACG	RT-qPCR
Gapdh R	CTCGCTCCTGGAAGATGGTG	RT-qPCR
Rosa26 Mutant	GCGAAGAGTTTGTCCTCAACC	Genotyping
Rosa26 F	AAAGTCGCTCTGAGTTGTTAT	Genotyping
Rosa26 R	GGAGCGGGAGAAATGGATATG	Genotyping
S100β F	GGTGACAAGCACAAGCTGAA	RT-qPCR
S100β R	TCCATCACTTTGTCCACCAC	RT-qPCR
Sox11 F	CCCTGTCGCTGGTGGATAAG	RT-qPCR
Sox11 R	GGTCGGAGAAGTTCGCCTC	RT-qPCR
Tgfβr3 F	GTCCCTGTGTTTGTCCTGATG	RT-qPCR
Tgfβr3 R	AGCCAGACAGAACGGTGAAG	RT-qPCR

Unless otherwise stated, RT-qPCR primers were newly designed and validated for these experiments. Genotyping primers for E2f1 (O'Hare et al.,

2000), E2f3 (Wu et al., 2001), Nestin-Cre-ER^{T2} (Chow et al., 2008), and YFP (Srinivas et al., 2001) are from previously established models. RT-qPCR primer sequences for Gapdh (Karuppaiya et al., 2017), E2f1 (Chen et al., 2013), E2f3 (Chen et al., 2013), and Akna mRNA (Camargo Ortega et al., 2019) have been previously published.