The Role of Activating E2Fs in Neural Stem Cell maintenance from Development to Adulthood

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ABSTRACT

The recent discovery of adult neural precursor cells (NPCs) in the dentate gyrus and the subventricular zone of the lateral ventricles of most mammals holds much hope for the potential regeneration of damaged brain tissue. However, their use has been limited by their low numbers and relatively quiescent state, particularly in the aging brain. Previous studies from our laboratory have demonstrated a crucial role for the Rb/E2F pathway in the regulation and proliferation of NPCs, and the direct mechanistic involvement of E2F3 in regulating the pluripotency factor, Sox2. More recently, our investigations into the roles of E2F1 and E2F3 in during adult neurogenesis have revealed that loss of both these genes results in a dramatic loss of adult NPCs. Here, we have employed the Emx1-Cre and Nestin-CreER^{T2} transgenic models, to specifically delete E2F1 and E2F3 in the cerebral cortex and in NPCs in order to investigate the role of both these genes in embryonic neurogenesis. Our results suggest a switch in the requirement for both E2Fs 1 and 3 between embryonic and adult NPCs, demonstrated by a decrease in NPC proliferation and numbers starting only during late embryonic development and persisting through postnatal neurogenesis. These findings suggest that E2Fs 1 and 3 are essential for the maintenance of stem cells and neurogenesis in the adult brain. Moreover, their deletion results in defects in learning and memory. These studies reveal a crucial role for activating E2Fs in the long-term maintenance and proliferation of neural stem cells.

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LIST OF ABBREVIATIONS

Ascl1 Achaete-scute homolog 1

ANOVA Analysis of Variance

BrdU 5-bromo-2-deoxyuridine

Cdk Cyclin-dependent-kinase

Cre Cyclization recombination enzyme

DAPI 4',6-diamidino-2-phenylindole

Dcx Doublecortin

DG Dentate Gyrus

DKO Double-Knockout

DP Dimerization Partner

E Embryonic-day

E2F E2 promoter binding factor

Emx1 Empty Spiracles Homeobox 1

ER^{T2} Tamoxifen regulated estrogen receptor ligand binding domain

FGF2 fibroblast growth factor 2

Flox Flanked by LoxP sites

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

Ki67 Antigen Ki-67

KO Knockout

Mash1 Mammalian Achaete Scute Homolog 1

MEFs Mouse Embryonic Fibroblasts

mL milliliter

min minute

MWM Morris Water Maze

NPC Neural Precursor Cell

NSC Neural Stem Cell

P Postnatal day

p53 Tumor protein 53

p107 Retinoblastoma like protein 1

p130 Retinoblastoma like protein 2

PBS Phosphate buffered saline

PFA Paraformaldehyde

qPCR quantitative Polymerase Chain Reaction

Rb Retinoblastoma protein

RGC Radial Glial Cell

SDS sodium dodecyl sulphate

sec second

SGZ Subgranular zone

Sox2 Sex determining region Y box 2

SVZ Subventricular zone

TAM Tamoxifen

Tbr2 T box brain gene 2

TKO Triple knockout

µL Microliter

µm Micrometer

WT Wild-type

VZ Ventricular zone

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INTRODUCTION

Cortical development relies on exquisite co-ordination of the cell cycle, specifically in maintaining a population of neural precursor cells (NPCs). Previous studies from the Slack laboratory have demonstrated a crucial regulatory role for both the Rb Family of pocket proteins, as well as their main binding partners, the E2 promoter binding factor (E2F) family of transcription factors, during developmental neurogenesis. Cell cycle regulators have been shown to orchestrate several types of cell division involved in maintaining a pool of neural stem-like cells throughout neurogenesis; these include proliferation, differentiation and self-renewal (Yoshikawa, 2000).

It was previously believed that a newborn neuron could only be generated during embryogenesis. However, the dogma that the adult brain cannot generate neurons from progenitor cells was disproven during the past few decades, when a persistent population of NPCs capable of neurogenesis was revealed in mammals (Altman, 1962). Neurogenesis is known to take place from embryogenesis to adulthood and is reliant on the size and maintenance of a persistent NPC population throughout life. The balance between the stages of quiescence, proliferation, differentiation, and apoptosis is key to stable growth and development, and is necessary to maintain a quiescent pool of cells to prevent the exhaustion of the NPC required for neurogenesis specifically during adulthood or in case of neurogenic disease.

Adult neurogenesis occurs in two locations in the mammalian brain (Ming and Song, 2011): the subventricular zone (SVZ) lining the lateral ventricles (Reynolds and Weiss, 1992) and the dentate gyrus (DG) of the hippocampus (Gage et al., 1995). In

1998, it was discovered that the DG of adult humans possesses the ability to generate new neurons (Eriksson et al., 1998). The presence of adult NPCs, which are defined here to refer to both stem and progenitor populations, and their continual generation of newborn neurons, holds much hope for the potential therapy of the damaged brain. However, their use has been limited, as the mechanisms underlying their proliferation and survival have yet to be determined. In order to have a better grasp on neurogenesis in the adult brain, the molecular pathways regulating neurogenesis during development need to be better understood. Studies using mouse models deficient for individual cell cycle genes have shown that several genes are in fact crucial for proliferation in the developing nervous system, are involved in differentiation, and have roles beyond cell cycle control (McClellan and Slack, 2006).

The goal of this introduction is to provide an overview of the requirement for the Rb/E2F pathway during embryonic and postnatal neurogenesis in regulating and maintaining a neural precursor population throughout multiple stages of development. The central theme of this thesis is to elucidate the role of activator E2Fs in the maintenance of neural precursor cells during neurogenesis, from development to adulthood.

1.1. Rb/E2F Pathway

1.1.1. Rb and Pocket Proteins

The retinoblastoma (Rb) protein, a gene mutated in malignant tumors of the retina and the first tumor suppressor gene to be cloned (Dryja et al., 1986, Friend et al., 1986, Lee et al., 1987) acts as a regulator for the G_1-S phase checkpoint of the cell cycle (McClellan and Slack, 2006, Chen et al., 2009b). Alongside two other closely related genes, Retinoblastoma like protein 1 (p107) and Retinoblastoma like protein 2 (p130), they form the Rb family of pocket proteins. These three Rb family members exhibit sequence homology in their A/B pocket domain responsible for binding E2Fs, and play a major role in the maintenance of cell cycle stability.

Each pocket protein carries a characteristic and unique expression profile, linked to different stages of the cell cycle, and possesses distinct roles. Rb is expressed throughout G0/G1/S phases of the cell cycle, while p107 is mostly evident at the late G1 and S phases (Shirodkar et al., 1992). p130 is more commonly expressed in cells undergoing quiescence (Cobrinik et al., 1993, Shin et al., 1995), and forms complexes with E2F transcription factors during the quiescent G0 and early G1 phases (Smith et al., 1996). Rb expression is evident in dividing as well as post-mitotic NPCs (representing all mitotic neurogenic cells), in addition to being expressed in quiescent neurons, while p107 is only up-regulated in non-differentiated cells, including the highly proliferative cells that constitute the Ventricular Zone (VZ) of the developing brain (Shin et al., 1995, Callaghan et al., 1999, Ferguson and Slack, 2001, Vanderluit et al., 2004).

The pocket protein family has a crucial role in cell proliferation: the Rb gene is a

known regulator of proliferation (Hurford et al., 1997, Classon et al., 2000) since deleting it results in accelerating the G1 phase of the cell cycle, whereas overexpressing Rb leads to cell cycle arrest in mouse embryonic fibroblasts (MEFs) (Goodrich et al., 1991, Qin et al., 1992). In many cell types, overexpression of all three pocket proteins causes cell cycle arrest at the G1-phase of the cell cycle, a phenotype that can be rescued by co-expression and interaction with the E2Fs, which has been shown in osteosarcoma and human primary T cell lines (Qin et al., 1995, Vairo et al., 1995, Moberg et al., 1996). Triple-knockout (TKO) MEFs displayed shorter cell cycles than wild-type (WT), did not undergo senescence and were resistant to G1 arrest signals, highlighting the fundamental role of these genes in the G1/S transition phase (Sage et al., 2000).

The presence of more intense phenotypes in compound knockouts than in single nulls suggests the presence of functional redundancy among pocket proteins (Hurford et al., 1997). Exacerbated proliferative defects in mice lacking all three pocket proteins indicate that these genes could partially compensate for each other, as TKO animals have a much more severe proliferative defect than Rb knockouts alone (Wirt et al., 2010). Developmentally, TKO embryos terminate between embryonic day (E) 9-11 (Wirt et al., 2010), while Rb-deficiency is embryonic lethal between E12.5-15.5 (Jacks et al., 1992). Interestingly, germ-line knockout of p107 does not cause lethality and did not show abnormalities, however, p130-deletion is lethal between E11-13 specifically on a Balb C genetic background which has been mechanistically linked to hypoxic stress and skeletal muscle defects, but not on C57BL/6 background (Cobrinik et al., 1992, Cobrinik et al., 1996, Lee et al., 1996, LeCouter et al., 1998). Double knockout (DKO) of p107/p130 lead to bone and cartilage malformations that result in immediate postnatal death

(Cobrinik et al., 1996), while DKO of Rb/ p130 result in death early during development, and homozygous deletion of both Rb and p107 results in termination at E11.5 (Lee et al., 1996, Sage et al., 2000). For these reasons, inducible Cre-recombinase/Flox knockout techniques for both Rb and p130 have been developed. More recently, it was shown that in the absence of Rb or both Rb and p130, there is an upregulation of p107 during late retinal development that prevents ectopic proliferation, yet it is haploinsufficient to rescue the phenotype of Rb/p130 double-knockout (Ajioka et al., 2007). In summary, these studies demonstrate that the members of the pocket proteins family have the ability to compensate for one another.

Rb is known to interact with E2Fs1-4, whereas p107 and p130 preferentially form complexes with E2Fs 4 and 5 (Hurford et al., 1997). Rb interacts with E2F genes through an A/B binding pocket domain, regulating the cell cycle at the G1/S checkpoint in many cell types (Cobrinik et al., 1992, Hamel et al., 1992, Sherr, 1993, Harbour and Dean, 2000, Cobrinik, 2005). Pocket proteins are regulated by phosphorylation: for example, the hypo-phosphorylated Rb represses E2Fs through binding, while inactive pRB, which is phosphorylated by cyclin-dependent kinases (Cdk), releases the E2Fs allowing activation of downstream targets hence leading to cell cycle progression and DNA synthesis, as shown in Figure 1 (Chen et al., 1989, DeCaprio et al., 1989, Dyson, 1998, Harbour and Dean, 2000). In addition, the Rb/E2F complex in itself can act as a transcription repressor (Sellers et al., 1995, Harbour and Dean, 2000), and direct interactions between E2Fs and the pocket protein family can regulate gene expression at E2F responsive sites (Chen et al., 2009b).

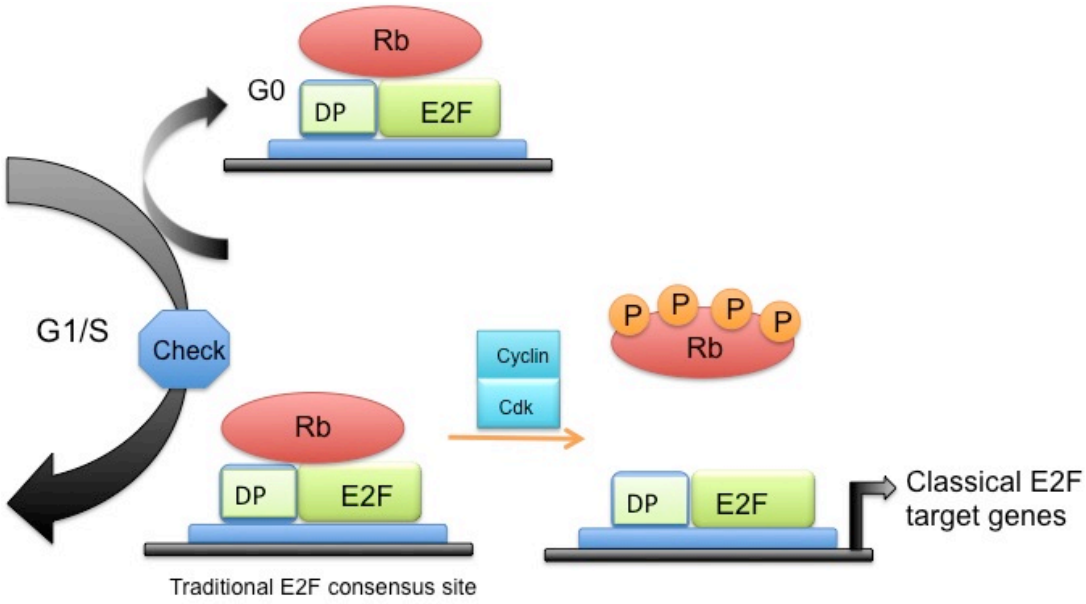

Figure 1. The Role of Rb/E2F pathway in cell cycle regulation:

Active hypophosphorylated Rb binds and represses E2F transcription factors. Inactivation of Rb by Cyclin/Cdk complexes, dissociates it from activator E2F-DP complex, allowing transcription of the E2F target genes leading to entry of the cell into S phase and cell cycle progression. Adapted from (McClellan and Slack, 2006).

1.1.2. E2F Family

E2Fs were first recognized by a sequence element known to bind the adenovirus E1A inducible E2 promoter DNA consensus sequence (Kovesdi et al., 1986, Kovesdi et al., 1987). The role of the E2F family is complex, as each of its members is known to have distinct functions based on the presence or absence of Rb. This transcription factor family includes eight genes; they include classical E2Fs 1-6, which heterodimerize with the dimerization partner DP (DP1-3) genes to become active and affect genes essential for both DNA replication (e.g. thymidine kinase, dihydrofolate reductase, DNA polymerase-α) and cell cycle regulation (Trimarchi and Lees, 2002). Furthermore, E2Fs 7-8 are considered 'atypical E2Fs', as they bind DNA in a DP-independent manner (Christensen et al., 2005). The E2F3 gene possesses two isoforms, E2F3a and E2F3b, which can display unique expression patterns despite possessing similar DNA binding domains as well as having the same transactivation and pocket protein binding pattern (Julian et al., 2013). E2F3b is missing a ubiquitin targeting domain at its N-terminus, which results in a distinctive coding exon and makes it the shorter isoform (Leone et al., 2000) (Figure 2).

Of the classical E2Fs, E2F1, E2F2 and E2F3a are traditionally considered activators, due to their activation of gene transcription along the G1-S phase (Wu et al., 2001), whereas E2F3b, E2F4, E2F5 and E2F6 are considered repressor E2Fs (Frolov et al., 2001, Trimarchi and Lees, 2002, Chen et al., 2009b). Only E2Fs 1-5 necessitate an interaction with pocket proteins for function, as they contain a trans-activation domain at the C-terminus, which contains a binding site for pocket proteins (Chen et al., 2009b) (Figure 2). As previously discussed, E2F transcription factors have shown different

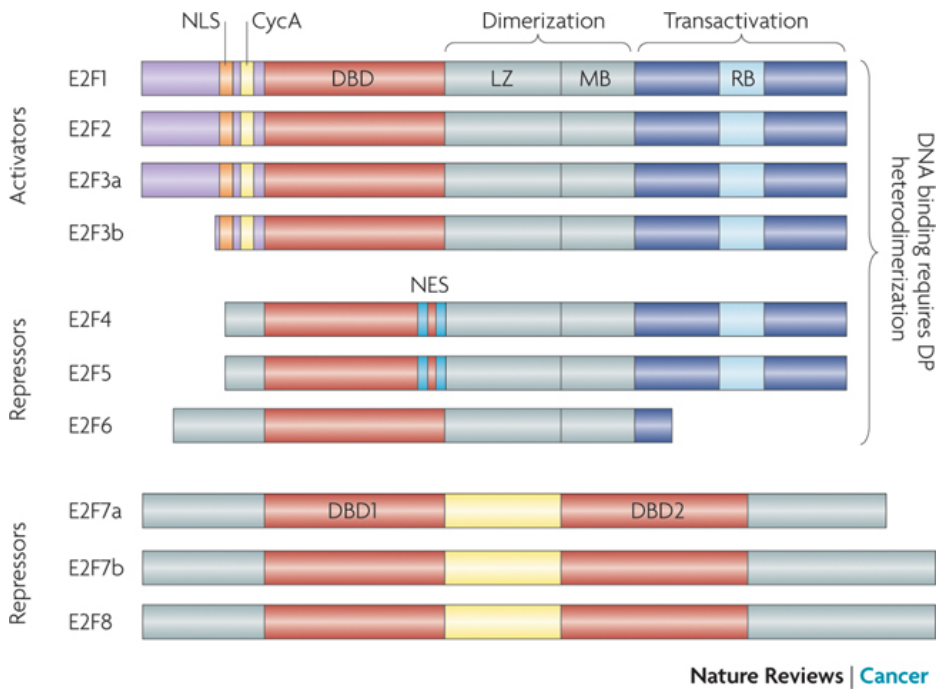

Figure 2. The members of the E2F family of transcription factors:

The eight members of the E2F family are divided into three categories: activators, repressors, and 'atypical' repressors. All E2Fs have a DNA binding sequence (DBD) allowing activation or repression of specific target genes. Only E2F1-6 require Dimerization Partners (DP) and Rb-dependent binding. Modified from (Chen et al., 2009b).

binding preference to different pocket proteins during the various stages of the cell cycle (Cobrinik et al., 1993, Chen et al., 2009b).

1.1.2.1. Activator E2Fs

For the purpose of this study, we will focus mainly on activator E2Fs, E2F1-3, which were traditionally believed to be indispensable for cells to undergo proliferation (Wu et al., 2001). E2F1 has unique roles *in vivo,* which include being a tumor suppressor and being involved in cell death (Field et al., 1996, Yamasaki et al., 1996). The tumor suppressor role attributed to E2F1 was identified by the development of several forms of cancer in E2F1 deficient mice, including reproductive tract sarcoma, lung tumors, and lymphoid tumors (Field et al., 1996, Yamasaki et al., 1996), whereas its importance in initiating cell death was suggested by the increased number of T-lymphocytes, originally caused by faulty apoptosis during thymocyte maturation, in E2F1 knockout (KO) mice (Field et al., 1996).

Several other studies have attributed pro-apoptotic roles to E2F1, by inducing cells with proliferation defects past the G1-S phase checkpoint, and leading to faulty cell division followed by apoptosis (Field et al., 1996, Shan et al., 1996, Tsai et al., 1998). Furthermore, overexpressing E2F1 leads to cell death in fibroblast cells (Wu and Levine, 1994). E2F1 is known to induce cell death both in a Tumor protein 53 (p53)-dependent and -independent manner (Wu and Levine, 1994, Holmberg et al., 1998). In cultured cortical neurons, E2F1 has been shown to modulate apoptosis through a caspase-3 dependent cascade that leads to DNA fragmentation. E2F1 null cells had a lower caspase-3-like activity, thus are resistant to cell death (Hou et al., 2000) A pro-apoptotic protein

called SIVA, which is upregulated following stroke injury, is a direct E2F1 and p53 target in neuronal cells (Fortin et al., 2004). Damage in DNA of apoptotic neuronal cells, promotes Cdk activation, leading to the inactivation of Rb and unbound E2F activity. This phenotype was rescued via blockade of E2F activity, protecting cortical neurons against cell death (Park et al., 2000). E2F1 also governs cell death in a p53-independent manner through induction of beta amyloid toxicity and potassium deprivation in neuronal cells (Giovanni et al., 2000, O'Hare et al., 2000). Furthermore, loss of E2F1 prolongs the survival of Rb-mutant embryos, highlighting the pro-apoptotic role of E2F1 in correlation with Rb (Tsai et al., 1998). Additionally, Rb/E2F1 double knockout mice had suppression of p53 up-regulation (Tsai et al., 1998). These studies highlight the importance of E2F1 in cell death, specifically in the context of neuronal cells.

E2F3 is crucial for normal development, as mice null for both isoforms of this gene die perinatally, linked mechanistically to cardiac malformations and growth retardation (Humbert et al., 2000b, Cloud et al., 2002, King et al., 2008). MEFs isolated from $E2F3^{-/-}$ mice, show a reduction in E2F-responsive genes, resulting in defects in DNA synthesis, suggesting a critical role for E2F3 in mediating normal cell cycle-dependent activation of these genes.

Confirming the role of transcriptional regulation by the Rb/E2F pathway, inactivation of E2F1 and E2F3 rescues the aberrant E2F activity that results from Rb deletion (Chen et al., 2007, McClellan et al., 2007). The two isoforms of E2F3 appear to be having overlapping functions as only one of them is required for normal development in the absence of the other activators E2F1 and E2F2 (Tsai et al., 2008)

E2Fs often have redundant roles and compensatory mechanisms (Xu et al., 2007, Tsai et al., 2008, van den Heuvel and Dyson, 2008). The functional overlap of activating E2Fs *in vivo* has also been suggested following a study done by Cloud and colleagues (2002). This study has shown that E2F3 mutant mice suffer premature death resulting from congestive heart failure, proving the essential role of E2F3 in embryonic, neonatal and adult viability, which are different phenotypes from the ones observed in E2F1-null mice, promoting unique roles for both proteins. Even though a viable double knockout model for both E2F1 and E2F3 could not be generated, $E2F1^{-/-}$ $E2F3^{+/-}$ mice suffered more severe developmental phenotypes than single knockouts, advocating for a potential functional overlap of E2F1 and E2F3 in different tissues (Cloud et al., 2002).

In vitro, deletion of E2F1, E2F2 and E2F3 causes cell cycle arrest and loss of proliferation in MEFs, in addition to reduced expression of G1/S regulating genes (Wu et al., 2001). Several other studies have shown that without activator E2Fs, there is an arrest of the cell cycle, again advocating for the crucial role of these E2Fs for entry into the S phase (Sharma et al., 2006, Timmers et al., 2007, Wenzel et al., 2011). Activator E2Fs are predominantly expressed in actively dividing cells and their deregulation results in proliferative defects as over-expression of these genes pushes quiescent cells to re-enter the cell cycle (Qin et al., 1994, Asano et al., 1996).

More recent *in-vivo* studies have shown, however, that cell cycle progression is possible in the absence of the three traditional activator E2Fs (Chen et al., 2009a, Chong et al., 2009, Wenzel et al., 2011). A 2009 study demonstrated that proliferation in the retina can occur without E2F1-3 However E2F1-3-deficient progenitor cells eventually undergo cell death due to a deregulation in p53 (Chen et al., 2009a). The same group

revealed similar results in stem and progenitor intestinal cells, showing that proliferation is unaffected by the lack of all three activator E2Fs and that E2F1-3 function as cell cycle repressors in differentiating cells, while interacting with Rb, to enable cell cycle exit and inhibit E2F target genes (Chong et al., 2009). Furthermore, a later study supports the hypothesis that activator E2Fs are not crucial for proliferation, while it shows they are essential for normal development and differentiation as they play a repressive role in the mouse lens (Wenzel et al., 2011). These findings advocate for a potential switch in role between activation and repression of the cell cycle for activator E2Fs in the presence of Rb, as it has been shown that they can function as transcriptional repressors through recruitment of Rb to target promoters (Chen et al., 2009a, Chong et al., 2009, Wenzel et al., 2011).

1.1.2.2. Repressor E2Fs

E2Fs 4-8 are highly expressed in quiescent and post-mitotic cell populations, suggesting that they are responsible for keeping cells in a non-proliferative state, hence the name "repressor" E2Fs (Wu et al., 2001, Trimarchi and Lees, 2002, Chen et al., 2009b). Absence of E2Fs 4 and 5 leads to the inability of MEFs to exit cell cycle *in vitro* despite the presence of cell cycle arrest signals, suggesting a role in proliferation repression for these genes (Bruce et al., 2000, Gaubatz et al., 2000). Furthermore, overexpressing these genes does not overcome cell cycle arrest as opposed to activator E2Fs (Ikeda et al., 1996, Moberg et al., 1996).

Repressor E2Fs have been shown to be crucial during development. Deletion of E2F4 causes severe defects notably in the ventral telencephalon, hematopoietic lineages

and gut epithelium during embryonic development, which leads to lethality in early postnatal mice (Humbert et al., 2000a, Rempel et al., 2000, Ruzhynsky et al., 2007). These studies highlight the importance of E2F4 for controlling maturation and normal brain development, specifically through control of the Sonic Hedgehog pathway for the ventral telencephalon development (Ruzhynsky et al., 2007). E2F5-deficient mice exhibit hydrocephalus due to an excessive secretion of cerebrospinal fluid, showing the dispensability of this repressor in proliferation, despite its crucial tissue-specific role in differentiated cells of the brain (Lindeman et al., 1998). E2F6 is required for normal skeletal and testicular development, as abnormalities in these tissues are observed in $E2F6^{-/-}$ animals (Storre et al., 2002).

Atypical E2Fs, which regulate transcription in an Rb-independent manner, can be considered repressors since E2F7 has been shown to interact and down-regulate several E2F-responsive genes leading to arrest of the G1 phase of the cell cycle (Blais and Dynlacht, 2004, Li et al., 2008, Chen et al., 2012, Ouseph et al., 2012). Atypical E2Fs are most highly expressed during S/G2 transition phase of the cell cycle (Di Stefano et al., 2003, Christensen et al., 2005). Deletion of both atypical E2Fs leads to apoptosis, resulting in embryonic lethality at E11.5, as well as disrupted expression of their known target, E2F1, at the S/G2 transition (Li et al., 2008). E2F7 and 8 null MEFs exhibit accelerated S-phase, while overexpressing E2F7 inhibits S-phase entry in quiescent cells (Li et al., 2008, Westendorp et al., 2012). Deregulation in E2F7 levels at the G2/M transition does not cause any disruption in the E2F target genes, suggesting that the role of atypical E2Fs in S-phase progression is through modulation of E2F target genes at the G1/S phase (Westendorp et al., 2012).

In summary, the Rb/E2F pathway is a major regulator of the cell cycle and has been broadly studied in a variety of contexts, namely proliferation, differentiation and cell death. In the next section, formation of the mammalian cerebral cortex, which is a process that occurs in a highly intricate and organized manner, will be discussed. The fundamental processes of neurogenesis, which includes proliferation, differentiation, and migration, is governed by a coordination of numerous genetic pathways, including the Rb/E2F pathway (Gotz and Huttner, 2005). The goal of the next sections is to provide an overview of neurogenesis and cortical development, focusing on the involvement of cell cycle regulators Rb and E2Fs.

1.2. Cortical Development: Overview

The Central Nervous System originates as a layer of cells made up of primary precursors known as neuroepithelial cells, which represent the earliest stem cells in the brain (Merkle and Alvarez-Buylla, 2006). The neuroepithelium of the embryonic forebrain gives rise to the fully developed adult cerebral cortex through precise and tightly regulated cell division and migration events (Noctor et al., 2004, Stern, 2005). Cortical growth is dependent on neurons originating from radial glial cells (RGCs) residing in the ventricular zone (VZ), and indirectly from the intermediate progenitor population residing in the SVZ (Noctor et al., 2004). The cerebral cortex is a highly organized structure in the brain of mammals that regulates multiple cognitive functions (Sun and Hevner, 2014). It is composed of projection neurons and interneurons organized in horizontal layers (Rakic, 2009). While neurogenesis persists in two regions of the adult

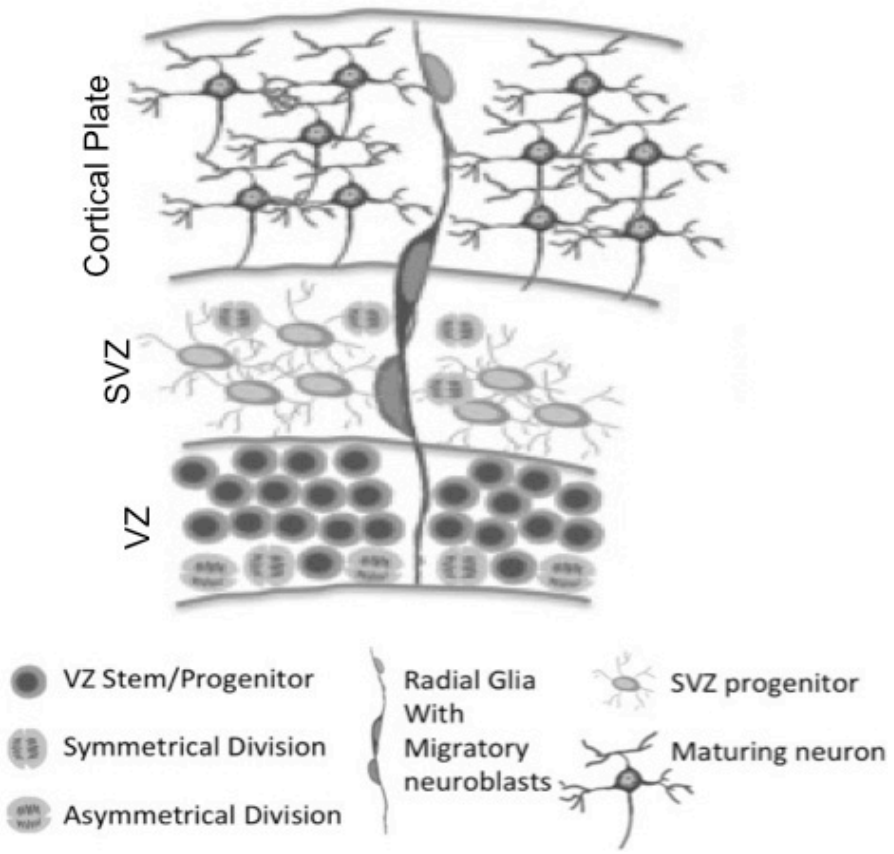

Figure 3. Cell divisions and types in the developing neocortex:

Apical precursor cells near the ventricular zone (VZ) are more multi-potent and undergo two types of divisions: symmetrical divisions to replenish themselves, and asymmetric divisions to give rise to more committed basal progenitors which migrate to upper layers of the subventricular zone (SVZ) and cortical plate. Modified from (Miranda, 2012).

brain, most neurons are born during embryogenesis. This next section will briefly describe neurogenesis in the cerebral cortex, involving cell division events, migration and differentiation of neural precursors.

1.2.1. Formation of the six-layered cortex

The telencephalon is the most complex region of the mammalian brain, and governs motor coordination and higher cognition among other crucial functions. This structure comprises the cerebral cortex and the basal ganglia, and possess a highly organized structure that includes several subdivisions, each with a unique cell population (Guillemot, 2005). The pool of precursor cells in the brain includes neuroepithelial multipotent stem cells that have the ability of self-renew through symmetric divisions and give rise to more stem-like cells, or differentiate into more committed progenitor cells via asymmetric divisions (Chenn and McConnell, 1995, Rakic, 1995, Yoshikawa, 2000, Miyata et al., 2004, Noctor et al., 2004) (Figure 3).

The telencephalon originates from neuroepithelial cells that form the wall of the neural tube in the embryonic ectoderm layer. This single layer of neuroepithelium gives rise to the earliest-born neurons of the cortex through the first asymmetric division, marking the onset of neurogenesis in the mouse at E11 (Takahashi et al., 1995). As the cortex develops, RGCs are produced around E12.5, and in turn give rise to basal, or non-surface dividing, progenitors cells (Miyata et al., 2004, Noctor et al., 2004, Gotz and Huttner, 2005). These events concur with the first wave of radial precursor cell migration by neural translocation from the VZ to the preplate. A second wave of cells results in the formation of the cortical plate layer, that is split into an outer marginal zone and an inner

sub-plate (Figure 4) (Angevine and Sidman, 1961, Bjornsson et al., 2015). Neurons are known to rely on RGCs as a scaffold for migration (Noctor et al., 2001, Boekhoorn et al., 2008). RGCs, the major type of NPCs during development, mostly undergo asymmetric divisions to produce two daughter cells, one of which is another RGC and the second being either a progenitor cell or a neuron (Noctor et al., 2001). In contrast, basal progenitor cells mainly divide symmetrically away from the VZ, forming the SVZ layer and eventually giving rise to two neuronal cells (Noctor et al., 2004). Corticogenesis continues until E17.5 (Takahashi et al., 1995). A developmental transition from neurogenesis to gliogenesis occurs near E18.5 in mouse, with the appearance of the first astrocytes from RGCs (Kriegstein and Alvarez-Buylla, 2009).

It has been established that cortical formation occurs in an inside-out manner. [^{3}H]-Thymidine-incorporation studies have shown that in the cerebral cortex, the outer layer consists of newly-born neurons whereas earlier born neurons reside in the innermost layers, notably the VZ and marginal zone (Angevine and Sidman, 1961, Rakic, 1972, 1974, 2009). As corticogenesis advances, more layers are generated, including the intermediate zone, the SVZ, the sub-plate, and the cortical plate, yielding a structure that contains 6-layers, through different modes of migration (Figure 4) (Kwan et al., 2012).

Newborn neurons exhibit four distinct phases of migration before eventually settling in the cortical plate (Noctor et al., 2004). Cells arising from asymmetric divisions of RGCs ascend rapidly from the VZ towards the SVZ, where they assume a multipolar morphology. The daughter cells remain in the SVZ for a day or more during the second stage migration. Following that, a retrograde movement characterizing the third phase occurs, where the cell-body moves towards the VZ. After contacting the ventricle, cells

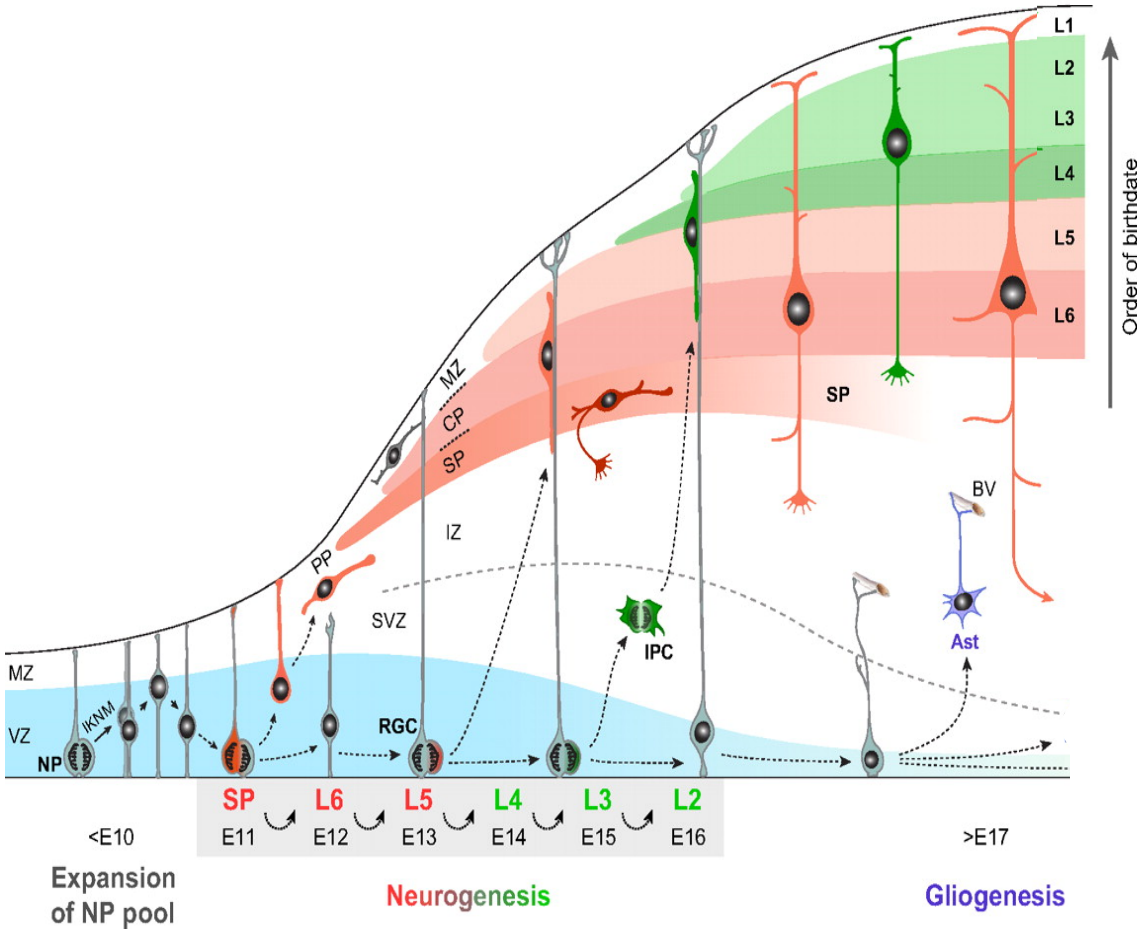

Figure 4. The formation of the 6 layered neocortex in the mouse from E10 to E17: Before the onset of neurogenesis, Neural Progenitors (NPs) in the ventricular zone (VZ) undergo interkinetic nuclear migration (IKNM) and divide symmetrically. At E11.5, NPs begin asymmetric division in order to generate neurons, which migrate in an inside-out manner, guided by radial Glial cells (RGC) to reach the upper layers. Earlier born neurons reside near the ventricular zone (VZ); while most-recently born ones migrate to the upper layer known as the pre-plate (PP). As development advances, more layers are generated, including the subventricular (SVZ), sub-plate (SP), fiber layer (FL), (CP), cortical plate and marginal zone (MZ). By the end of E17, the 6 layers of the cerebral cortex are formed. After E17.5, gliogenesis starts and NPs give rise to astrocytes (Ast). BV, blood vessel; E, Embryonic day; IZ, intermediate zone. Adapted from (Kwan et al., 2012).

become bipolar and migrate towards the cortical plate, undergoing their fourth migration (Noctor et al., 2004)

In conclusion, NPCs undergo extensive levels of proliferation during the early stages of forebrain development. Neurons are produced near the lateral ventricles in the transient proliferative zone, and then migrate radially and tangentially across the intermediate zone to reach their proper location within the cerebral cortex following differentiation (Rakic, 2009). This pattern of highly organized series of migrations and cellular divisions is what results in the formation of the 6 layers of the cerebral cortex (Dehay and Kennedy, 2007), where the first-born neurons constitute the deeper layers V and VI, while more recently born cells migrate radially to reside in more superficial coats II and III (Takahashi et al., 1999, Rice and Curran, 2001).

1.2.2. Types of cells and their corresponding markers during cortical development

During the development of the cerebral cortex, the NPC pool is mainly comprised of three types of cells: apical precursors, basal progenitors and immature neurons (Guillemot, 2005). RGCs, also referred to as apical precursors due to their division at the surface of the ventricle, are more multi-potent and give rise to neurons in the lower layers (Dehay and Kennedy, 2007). They are the main generators of progenitor cells embryonically and throughout postnatal neurogenesis (Gotz and Huttner, 2005). For the purpose of this study, we will use the pluripotency gene, Sox2, to indicate the presence of apical precursor cells in the neural stem cell (NSC) pool (Ellis et al., 2004, Bani-Yaghoub et al., 2006).

Intermediate, or transit amplifying, progenitor cells arise from radial glia and divide away from the ventricular surface, hence the name "non-surface-dividing cells" or basal progenitors. These cells express a T-domain transcription factor, Tbr2 (Englund et al., 2005, Kowalczyk et al., 2009). Typically, basal progenitors are known to only undergo one to two mitotic cycles before producing neurons in upper layers of the cortex (Englund et al., 2005, Dehay and Kennedy, 2007).

The basal progenitors give rise to the final cell population discussed in this study: the newly-born neurons, following differentiation. These cells are known to express Doublecortin (Dcx) which is a microtubule-associated protein required for proper radial migration of neurons across the cerebral cortex, and is expressed in both migrating and differentiated neurons (Francis et al., 1999, Boekhoorn et al., 2008). The control of proper cortical development thus depends on the balanced proliferation and differentiation of the different type of precursor cells (Sun and Hevner, 2014).

1.2.3. Neurogenesis in the adult SVZ

In the adult brain, there are two regions capable of generating functional neurons from a pool of NPCs throughout life: the SVZ of the lateral ventricle and subgranular zone (SGZ) of the hippocampus (Ming and Song, 2011). In both these neurogenic zones, stem-like cells are generally referred to as type B cells, which give rise to transit-amplifying progenitors, also known as C cells, that will eventually differentiate into neuroblasts, or type A cells (Kriegstein and Alvarez-Buylla, 2009). Type A cells will tangentially migrate along the rostral migratory stream to integrate into the olfactory bulb

and differentiate into two types of interneurons: granule and periglomerular cells, leaving the SVZ (Doetsch and Alvarez-Buylla, 1996, Ming and Song, 2011).

Sox2 expression can be observed in postnatal neurogenic regions, hence it can be used to label type B cells (Ellis et al., 2004). In order to identify type C cells, transcription factors Dlx2 and Mash1, or Achaete-scute homolog 1 or Ascl1, can be used as markers (Doetsch et al., 2002, Parras et al., 2004). Ascl1 is typically expressed in proliferating adult as well as embryonic progenitor cells (Andersen et al., 2014), Furthermore, Dcx is commonly used to identify type-A cells (Rousselot et al., 1995, Francis et al., 1999).

1.2.4. Cell cycle length in cortical development

The neocortical laminar fate is highly dependent on the cell-cycle (Takahashi et al., 1999). The size of the pool of precursor cells fluctuates during neural development, relating to variations in cell cycle duration (Dehay and Kennedy, 2007). The neuronal expansion occurring during mid-embryonic neurogenesis can actually be attributed to the high proliferative rates of the onset of neurogenesis, and the shortening of the length of the G1 phase (Polleux et al., 1997). As these precursor cells differentiate, they undergo cell cycle exit and are not allowed to re-enter (Polleux et al., 1997). Studies have shown that progenitor cells undergoing differentiating divisions exhibit significantly increased elongation of the cell cycle than those undergoing symmetrical proliferative divisions (Calegari et al., 2005). Hence, the length of the cell cycle can be directly linked to the type of cell division, proliferative versus differentiative, resulting in different cell types and controlling the size of the stem cell population in the brain (Calegari et al., 2005). It

has been suggested that, in the context of the developing brain, cell cycle length of progenitor cells is longer than that of stem-like cells, however the length of the S phase is higher in apical than in basal precursors (Arai et al., 2011, Bragado Alonso et al., 2014).

In the adult brain, as many cells are cycling as are quiescent, and of those actively dividing cells, 10% are type B cells, 60% are type C cells and 26% are type A cells (Doetsch et al., 2002, Bragado Alonso et al., 2014). Type C cells are mostly cycling at any given time, with a unique cell cycle pattern that consists of a longer S phase and an unusually short G1 (Ponti et al., 2013). However, type A cells have an elongation of the G1 phase and a shorter S phase. This lengthening is not seen in the transition between B to C, but the significance of such differences remains unknown (Ponti et al., 2013). Thus, the stem-like type B population is mostly quiescent throughout adult neurogenic regions. Further investigations linking cell-cycle variations and stemness would be relevant to help understand how to maintain a balanced pool of stem cells throughout life.

1.2.5. Origin of Adult NPCs

Recent studies have shown that adult stem-like cells are born during embryogenesis, yet remain quiescent until they are signaled to undergo cell division in the adult brain (Fuentealba et al., 2015, Furutachi et al., 2015). It was recently established that type B cell precursors are born between E13.5-15.5 but remain in the quiescent state until they are reactivated postnatally (Fuentealba et al., 2015). Lineage tracing of type B cells using a retroviral barcode library revealed that adult NSCs share common precursors embryonically with cells in the cortex, striatum, and septum. This connection vanishes after E15.5, suggesting that regional specification of NSCs occurs

during early development. Postnatal type B cells hence are clonally related to embryonic NSCs (Fuentealba et al., 2015).

Consistent with these findings, another recent publication has shown that adult NPCs are derived from a population of randomly selected cells during early embryonic development that undergo slow cell division (Furutachi et al., 2015). Portions of embryonic NPCs slow their proliferation and become largely quiescent throughout adulthood. The majority of adult stem cells in the SVZ are derived from these slowly dividing cells that are born around E14 and express the Cdk-inhibitor p57. The slow division is thought to be the cause of the formation of this cell population, and deletion of p57 hinders the establishment of adult NPCs. Hence, it is important to understand the mechanism behind maintaining the population of slowly dividing cells which would form stem cells in the adult brain, which are essential for dealing with stress and neurodegenerative disease (Furutachi et al., 2015). The involvement of p57 as a regulator of adult stem cell populations suggests a role for cell cycle regulators in this process.

In conclusion, normal brain development is a complex process that is tightly regulated by the cell-cycle. The Rb/E2F pathway has been shown to have neurogenic-specific functions, which will be discussed below (Ferguson and Slack, 2001).

1.3. Rb /E2F pathway in neurogenesis

1.3.1. Rb and pocket proteins in neurogenesis

Multiple studies have described distinct and overlapping functions of Rb/E2F members in the development of the nervous system (McClellan and Slack, 2006). Deletion of the Rb gene results in embryonic death between E13.5 and E15.5 due to severe neurological and hematopoietic defects, which highlights how crucial this pocket protein family member is for development (Clarke et al., 1992, Lee et al., 1992, Ferguson et al., 2002). Typically, mice nullizygous for Rb show severe cell division defects and cell death throughout the developing brain (Clarke et al., 1992, Lee et al., 1992, Lee et al., 1994). Furthermore, mice lacking this gene show maturation defects in their nerve cells (Lee et al., 1994). This highlights a crucial requirement for Rb in neuronal cell survival and differentiation. Rb is also required for committed NPCs to undergo cell cycle exit (Slack et al., 1998, Callaghan et al., 1999).

Rb-deficient embryos exhibited massive neuronal loss, however this phenotype was rescued by the supply of a WT placenta to $Rb^{-/-}$ embryos, demonstrating that of Rb during development is crucial for neuronal survival with both cell autonomous and non-autonomous roles during development (de Bruin et al., 2003). To further understand the importance of this gene in cerebral cortex development, previously reported studies from our lab employed a conditional Rb mutant mouse that could survive until birth, using a Cre recombinase expressed from the brain factor 1 (Bf-1) promoter to delete Rb specifically within the telencephalon (Ferguson et al., 2002). Examination of this mouse during mid-cortical development at E15.5, showed enhanced proliferation of neuroblasts

marked by an increase of 5-bromo-2-deoxyuridine (BrdU) labeled cells in the cortical plate and the intermediate zone, caused by an ectopic entry into the S phase of the cell cycle. Hence, Rb-null brains exhibit enhanced neurogenesis and proliferation (Ferguson et al., 2002).

Consistent with these findings, conditional Rb-deficient embryonic NPCs demonstrated enhanced neuronal survival in the central nervous system relative to the whole body knockout (MacPherson et al., 2003). The conditional Rb-knockout showed the importance of this gene for normal brain formation, as the mutant mice exhibited an increased brain size and weight (Ferguson et al., 2002, MacPherson et al., 2003). These studies suggest that the proliferation defects exhibited by the lack of Rb are cell autonomous (Ferguson et al., 2002, MacPherson et al., 2003). Moreover, these brains show defects in the migration of interneurons (Ferguson et al., 2005). These data support a crucial role of the Rb gene in the regulation of proliferation and survival of neurons during mouse cortical development.

The Rb/E2F axis has been proven essential for normal maintenance of differentiation during forebrain development through the regulation of *Dlx1*/*Dlx2* bigene cluster. In the absence of Rb, repressor E2Fs bind and deregulate both *Dlx1* and *Dlx2* genes, impairing transcription and suppressing differentiation, which leads to migration defects in the mouse model (Ghanem et al., 2012).

Independent of Rb, another pocket protein family member, p107, also plays a crucial role in regulating NPCs in the mouse brain. Adult as well as embryonic p107-null mice exhibit an enhanced stem cell population marked by an enhanced pool of slowly

dividing cells, which were labeled by long-term BrdU as rapidly dividing cells do not retain the stain as they differentiate and migrate away from the ventricular zone, when compared to controls. This phenotype was confirmed by an increase in the number of neurospheres from the p107 mutant adult and embryonic mice (Vanderluit et al., 2004). These neurospheres, which are a way to assess NPCs *in vitro,* showed a high self-renewal capacity, in addition to high levels of Notch pathway proteins. These results demonstrate the involvement of p107 in negatively regulating NPCs in both the developing and the adult brain possibly through an interaction with the Notch pathway. It was later suggested that p107 controls NPCs self-renewal and differentiation through repression a Notch protein, Hes1 (Vanderluit et al., 2007).

1.3.2. Activator E2Fs in neurogenesis

Previous research has shown that a deregulation in activator E2Fs causes proliferative defects. A 1999 study suggested that E2Fs 1 and 3 are linked to Rb-mediated regulation of the developing nervous system (Callaghan et al., 1999). Deletion of Rb showed that NPCs exhibited delayed differentiation *in vitro* as well as an increase in free E2F binding activity, which includes both E2F 1 and 3. An increase in cyclin A, cyclin E and Cdk-2, which are target genes for E2F1 and 3, in $Rb^{-/-}$ differentiated neurospheres, suggests that Rb deficiency leads to an enhanced proliferation of neural precursor cells and a deregulation in E2F binding activity. This study also indicated that no E2F2 expression has been detected in the brain or cortical progenitors (Callaghan et al., 1999).

Later, a 2002 study on mice lacking the E2F1 gene showed impairment in adult

neurogenesis, highlighting the importance of this gene in regulating neuronal proliferation. E2F1 mutant mice showed considerably lower numbers of proliferating cells, visualized by BrdU staining, in areas with stem cell activity such as the DG and SVZ, in addition to lower numbers of newborn neurons, when compared to the WT littermates (Cooper-Kuhn et al., 2002).

E2F3 has been proven essential for neural precursor proliferation and migration (McClellan et al., 2007). In mice lacking E2F3, BrdU staining revealed a decrease in numbers of neural progenitors lining the lateral ventricles when compared to controls from the same litter. Similar results were observed in the embryo. Moreover, a rescue experiment for the ectopic proliferation in the developing cortex of Rb-deficient mice has been done by single deletion of E2F1 and E2F3, suggesting that both of these activating E2Fs may be important regulators of NPC function. These findings show that E2F3 regulates neural precursor proliferation, and imply functional redundancy amid this transcription factor family. Furthermore, E2F3 was shown to modulate Rb-dependent migration, through regulation of the Neogenin gene, which is involved in neural migration (Andrusiak et al., 2011). Moreover, Chen and colleagues revealed that the E2F3a isoform is involved in Rb-mediated neuronal migration and differentiation in the retina (Chen et al., 2007).

The Rb/E2F pathway is also involved in mediating NPC proliferation and population through regulation of fibroblast growth factor 2 (FGF2) (McClellan et al., 2009). In Rb family-null brains, FGF2 is aberrantly upregulated due to a deregulated activity of the E2F3 protein. Through Chromatin immunoprecipitation assays, it has been demonstrated that the FGF2 gene is tightly regulated by p107 and E2F3. Progenitor

responsiveness assessments in p107 and E2F3 mutant mice suggest that both proteins are mediators of FGF2 in NPCs, whereby E2F3 transcriptional activation of FGF2 is repressed by p107. This regulates proliferation signals of NPCs *in vivo* (McClellan et al., 2009).

Earlier work from our lab has reported that E2F3 isoforms, E2F3a and E2F3b, differentially regulate embryonic and adult neurogenesis. The study, published in 2013, revealed that E2F3a regulates the expression of the pluripotency factor Sox2 in both embryonic and adult NPCs. Deletion of E2F3a leads to reduced embryonic and adult neurogenesis, in addition to defects in cognitive functions of adult mice. E2F3a KO also causes an increase in Sox2 expression and NPC expansion while decreasing the number of committed neurons (Julian et al., 2013). Conversely, deleting E2F3b shows the opposite effect, promoting neural precursor pool expansion, which suggests that a balance in both isoforms is essential in maintaining normal levels of Sox2, and neurogenesis (Julian et al., 2013). These data shows that E2F3 regulates Sox2, suggesting that the Rb/E2F pathway is involved in NPC cell fate decisions. Altogether, these studies demonstrate specific and critical roles for both activator E2Fs 1 and 3 in neurogenesis, beyond cell cycle regulation, including migration and cell fate.

Preliminary research from our lab demonstrates a crucial role for activator E2Fs, E2F1 and E2F3, in regulating the NPC populations in the adult mouse brain. Using a conditional Empty Spiracles Homeobox 1 (Emx1)-Cre E2F3, E2F1 germline knockout model, where Cre-recombination occurs during early embryonic neurogenesis at E10.5 (Simeone et al., 1992), we examined mice at 6-weeks post-birth. These mice exhibited a dramatic loss of NPCs due to loss of both E2Fs 1 and 3. Significantly lower levels of

uncommitted NPCs, marked by Sox2, as well as Dcx+ newborn neurons and Ki67+ proliferating cells, were observed in the DG of the adult brain of double-knockouts (DKO) as compared to their littermate controls (Figure 5A). DKO animals also showed a severe decrease in the dentate gyrus mass (Figure 5A). Furthermore, using an inducible Nestin-CreER^{T2}, deletion of E2F3 was induced in 6 week-old adult mice, which were examined a month later. These mice exhibited a dramatic reduction in neurogenesis demonstrated by a decrease in Dcx+ newborn neurons, and a reduction in the total number of proliferating cells, yet no changes were detected in the number of Sox2+ stem-like cells in DKO relative to control animals (Figure 5B).

In summary, the Rb/E2F pathway was shown to be essential for the regulation of cell division and processes governing neurogenesis, whereby deregulations in Rb/E2F pathway proteins cause abnormal brain development. Our unpublished data establish the importance of activator E2Fs 1 and 3, specifically in regulating cell proliferation and population during neurogenesis. Subsequently, in-depth study of the embryonic cortical development of this DKO mouse model was necessary to determine whether the onset of this phenotype is early during development or the requirement for activating E2Fs in neurogenesis is limited to the adult stage.

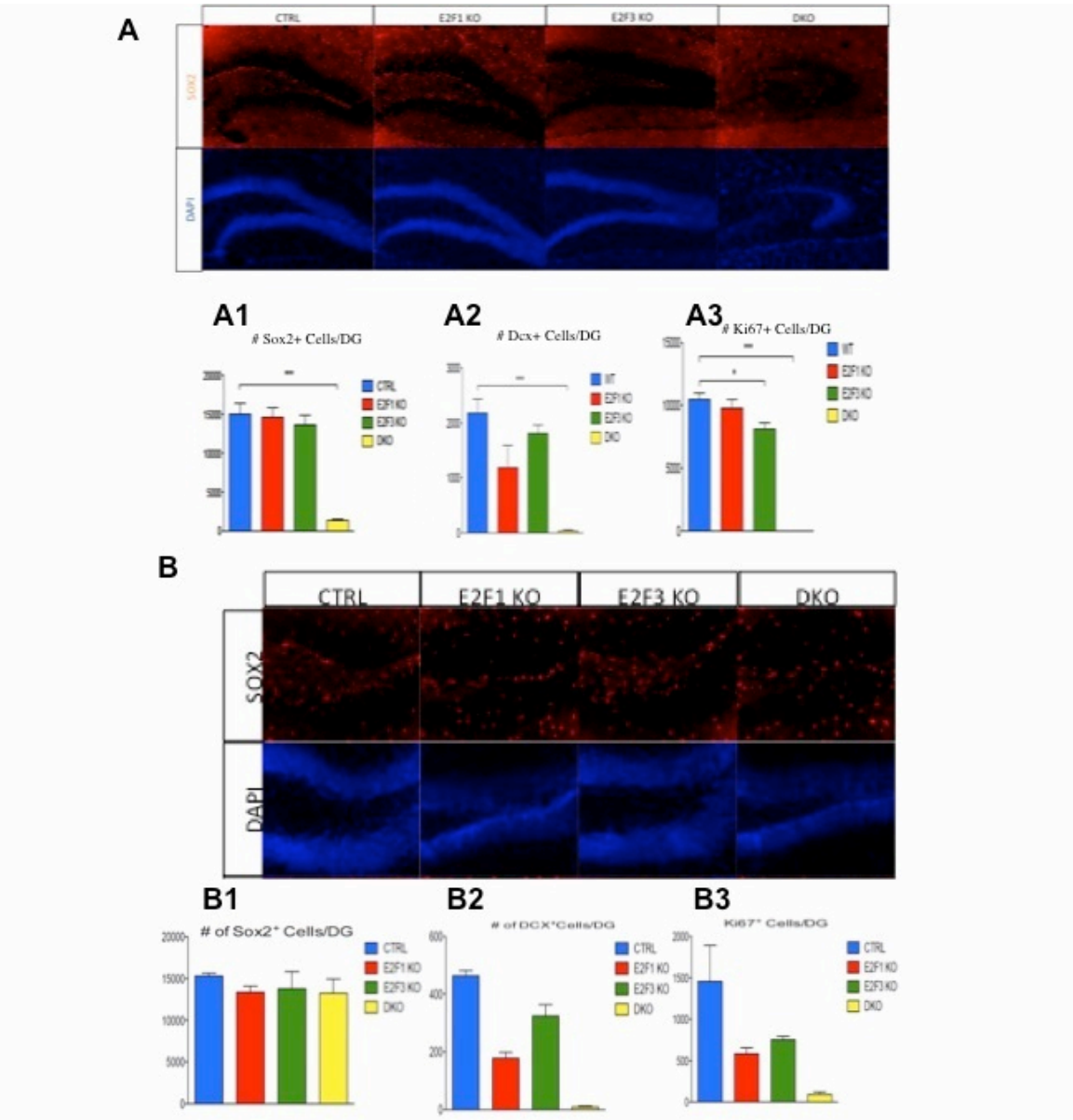

Figure 5. E2F1/3 DKO leads to decrease in NPC populations in adult mouse brain: **Panel A:** Immunohistochemistry against Sox2 (type B cells), counterstained with DAPI in 6 weeks old control ($E2F1^{+/+}$ $E2F3^{flox/flox}$), E2F1 KO ($E2F1^{-/-}$ $E2F3^{f/f}$), E2F3 KO ($Emx1\text{-}Cre^{+}$ $E2F1^{+/+}$ $E2F3^{f/f}$) and DKO ($Emx1\text{-}Cre^{+}$ $E2F1^{-/-}$ $E2F3^{f/f}$) per dentate gyurs (DG). Graphs A1, A2 and A3 represent the numbers of Sox2, Dcx and Ki67 cells per DG, respectively. ANOVA on coronal serial sections. (n=3 ***p<0.001).
Panel B: Immunohistochemistry against Sox2, counterstained with DAPI in Tamoxifen induced 6 weeks old control ($E2F1^{+/+}$ $E2F3^{f/f}$), E2F1 KO ($E2F1^{-/-}$ $E2F3^{f/f}$), E2F3 KO ($Nestin\text{-}CreER^{T2+}$ $E2F1^{+/+}$ $E2F3^{f/f}$) and DKO ($Nestin\text{-}CreER^{T2+}$ $E2F1^{-/-}$ $E2F3^{f/f}$) per DG. These animals were sacrificed 5 weeks later. Graphs B1, B2 and B3 showing Sox2+ cells Dcx+ cells and Ki67+ cells per DG, respectively. Mean ± SEM, n=2. Scale bar=25 μm. Ahmadi, Vandenbosch and Slack, unpublished.

1.3. Rationale and Hypothesis of the present study

All of the above studies suggest a critical role for the cell cycle regulatory Rb/E2F pathway in cortical development and neurogenesis. However, the specific requirement for activator E2Fs from development to adulthood remains unknown. Therefore, I hypothesize that **activator E2Fs are essential for the maintenance of stem cells and neurogenesis such that their absence results in loss of adult NPCs and a long-term behavioral phenotype.** To test this hypothesis, two objectives were established:

Aim 1: Characterization of the requirement for E2Fs during developmental and adult neurogenesis.

Aim 2: To define when E2F requirement occurs during development.

To address these questions, we utilized two transgenic mouse models and examined their NPC population in several stages of neural development. The first model was used to characterize brain development in the absence of E2F1 and E2F3. The second model was used to determine when the precise requirement for these activator E2Fs develops by specifically deleting E2Fs 1 and 3 at different times during development. To answer the question of the effect of absence of cell cycle activators on cognition, we examined the behavior of DKO mice during adulthood. These studies will help us better understand the role of these two genes for maintaining a pool of NPCs throughout life.

MATERIALS AND METHODS

2.1. Mice

Mice used for this study were bred from different transgenic lines: E2F1$^{-/-}$, E2F3$^{f/f}$, Emx1-Cre and Nestin-CreER^{T2}. They were maintained on a mixed genetic background (FVBN/C57BL6/S129) (Charles River Laboratories, Wilmington, MA)

The E2F1 knockout mice are a germline model, generated with a neomycin cassette insertion into both exons 3 and 4, resulting in deletion of the DNA binding and heterodimer regions (Field et al., 1996).

As E2F3 whole-body knockout mice are not viable (Cloud et al., 2002), we employed E2F3 flox mice, which were obtained from Dr G. Leone and generated with a triple LoxP vector system, that includes a short recombination arm, a medium fragment that includes the floxed exon 3, as well as a longer one spanning exons 4 and 5 (Wu et al., 2001).

Two Cre systems were used in this study: Emx1-Cre and Nestin-Cre estrogen receptor ligand binding domain (ER^{T2}). In the Emx1-Cre model (Simeone et al., 1992), Cre expression targets the neural precursor population during embryonic cortical neurogenesis: when crossed with the floxed E2F3 mice, recombination begins at around embryonic day E10.5 (Simeone et al., 1992), which concurs with the onset of neurogenesis.

The second Cre model, Nestin-CreER^{T2} was obtained from Dr. S. Baker (St. Jude Children's Research Hospital) (Lavado et al., 2010) where Cre expression is evident in

the Nestin-expressing cells of the developing as well as the adult brain. Thus, this model is useful to study NPCs, and the effect of gene deletion on neurogenesis in both the adult and developing mouse brain. The activity of the Nestin-CreER^{T2} is induced by Tamoxifen (TAM) administration (Lavado et al., 2010), thus to delete E2F3 in embryos, pregnant females were administered one 100 µL dose of 20 mg/mL TAM through oral gavage. Postnatal day P 21 mice were given three 100 µl oral gavage doses of 30 mg/mL, whereas adult 6-weeks old mice received 100 µl of 50 mg/mL TAM over the course of five days, to ensure Cre-recombination and gene knockout (Figure 13).

The animals used in experiments were bred as follows: females or males carrying homozygous floxed E2F3 alleles and heterozygous E2F1 alleles (E2F3$^{flox/flox}$; E2F1$^{+/-}$), were crossed with females or males carrying homozygous floxed E2F3, heterozygous E2F1 alleles and Cre (Nestin-CreERT2^{+} E2F3$^{flox/flox}$; E2F1$^{+/-}$) or (Emx1-Cre^{+}, E2F3$^{flox/flox}$; E2F1$^{+/-}$), to generate DKO (E2F1$^{-/-}$ E2F3 $^{f/f}$ Emx1-Cre $^{+}$) or (E2F1$^{-/-}$ E2F3 $^{f/f}$ Nestin-CreER$^{T2\,+}$) and controls (E2F1 $^{+/-}$ E2F3 $^{f/f}$).

All experimental protocols were approved by the University of Ottawa Animal Care Committee. All mice were genotyped using the Sigma Extract-N-Amp kit (Sigma) and primers designed around the E2F1, E2F3, and Cre loci (Table 1) according to the manufacturer's protocol.

	Genotyping primers
E2F1	E2F1-5 5'-GGATATGATTCTTGGACTTCTTGG-3' E2F1-3 5'-CTAAATCTGACCACCAAACGC -3' PGKB 5'-CAAGTGCCAGCGGGGCTGCTA AAG-3'.
E2F3	Primer A 5'-GTGGCTGGAAGGGTGCCAAG-3' Primer B 5'- TGAATCATGGACA GAGCCAGG-3' Primer C 5'-GATTGATTCTGGGTTGTCAGG-3'
Emx1-Cre	Cre 3B 5'–TGACCAGAGTCAT CCTTAGCG-3' Cre 5B 5'-AATGCTTCTGTCCGTTTGCC-3'
Nestin-CreER^{T2}	Cre 3 5'–TTGCCCCTGTTTCACTATCCAG-3' Cre 5 5'–TGCTGTTTCACTGGTTATGCGG-3'
	qPCR Primers
E2F1	E2F1-For 5'-CTGCAGCAACTGCAGGAGAG-3' E2F1-REV 5'-CTCCGAAAGCAGTTGCAGCTG -3'
E2F3	E2F3 For 5'-AAACGCGGTATGATACGTCCC-3' E2F3 Rev'- CCATCAGGAGACTGGCTCAG-3'
GAPDH	FOR 5'–ACTGAGGCTCTCAGGAACCA-3' REV 5'-AGAGCCTAAAAGGGCTCAGG-3'

Table 1. Primers used for genotyping and qPCR

Forward and Reverse primers used for genotyping and qPCR purposes to amplify regions unique to GAPDH, Emx1-Cre (Simeone et al., 1992), Nestin-CreER^{T2} (Lavado et al., 2010), E2F1 (Field et al., 1996), and E2F3 (Wu et al., 2001) loci

2.2. Tissue fixation and cryosection

Pregnant mice were euthanized with an injection of 1 mg/g Euthanyl (Supplied by: Animal and Veterinary Care Services, University of Ottawa) followed by cervical dislocation. Then, pups were dissected out and euthanized by decapitation. Heads were fixed and preserved in 4% paraformaldehyde (PFA) (pH 7.4), then washed in 1% Phosphate Buffered Saline (PBS) and transferred into a 20% sucrose and 0.01% sodium azide in 1% PBS solution, 24 hours later. Heads were frozen using Tissue-Tek O.T.C Compound (Sakura). Tissue was cut in serial coronal sections at 14 μm thickness and mounted onto Superfrost Plus slides (Fisher) then stored at -80°C for immunohistochemistry. Postnatal mice were euthanized with an injection of Euthanyl, then perfused using 1% PBS followed by 4% PFA. Their brains were dissected out and stored as described above. Tissue was cut in serial coronal free-floating sections of 30 μm.

2.3. Immunohistochemistry

Slides were left to dry at room temperature, then were washed three times in 1% PBS. Antigen retrieval was performed when necessary, through 15-minute incubation at 95-98°C in Dako Antigen Retrieval solution (Dako), before antibody incubation. Slides were then left to cool for 5 minutes, rinsed once with 1% PBS then incubated overnight at 4ºC in primary antibody diluted in 0.1% Triton 0.1% Tween in PBS solution (Table 2). The next day, slides were washed three times with 1% PBS and incubated at room temperature for 45 minutes with secondary antibodies, which were also diluted in 0.1% Triton 0.1% Tween in PBS. Tissue sections were then treated for 5 minutes with 1μg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma), in 1% PBS followed by three washes in

1% PBS (Table 2). Finally, the slides were coverslipped with Immunomount (Genetex) and imaged using Zeiss confocal microscope and Zen imaging software.

2.4. Cell quantification

For manual cell quantification, counts were performed using confocal images on four adjacent sections from each brain that had been leveled based on their rostral-caudal location using Cresyl violet staining. For total cell counts, values are expressed as per population or area. For proliferative cell quantification, values are expressed by cells co-labeled with the proliferation marker Ki67 and normalized to 100 cells within each of the four fields that were imaged and counted for each animal. An unpaired two-tailed Student's T-test was performed for all graphs with a minimum 95% confidence threshold and n=3 for both control and mutant animals.

2.5. Western Blot

Proteins from neurosphere cultures of E15.5 Emx1-Cre^{+} E2F3$^{f/f}$ and control animals were isolated. Protein was extracted using a 4% sodium dodecyl sulphate (SDS) lysis buffer then quantified through Bradford assay. Western blots were adapted from published protocol (Cregan et al., 1999). Equal amounts (10 μg) of total protein per sample were electrophoresed through 8% SDS-PAGE, then blotted on a nitrocellulose membrane. Primary antibodies were incubated overnight at 4°C for immunoblotting (Table 2). The secondary antibodies were incubated for one hour at room temperature. Actin antibody was used as loading control (Table 2). Blots were developed by chemiluminescence according to the manufacturer's instructions (ECL; Amersham Biosciences).

2.6. quantitative Polymerase Chain Reaction (qPCR)

Total RNA from brain tissue of E2F1 Wild-type (WT), $E2F1^{-/-}$ and $E2F3^{flox/flox}$ animals were extracted using Trizol (Ambion, Life Technologies) according to the manufacturer's instructions. Equal amounts (25 μg/μL) of total RNA, which were quantified by NanoDrop, were loaded per sample. The Rotor Gene Syber-green RT qPCR kit (QIAGEN) was used to amplify specific target genes (Table 1). All expression values were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. Program using Rotor Q gene qTPCR was as follows: hold for 10 mins at 55ºC for reverse transcription, then hold for 5 mins at 95ºC for initial PCR activation step, cycling for 40 cycles for 5 sec at 95ºC, which is the denaturation step, melt ramp 50 to 99ºC, rising 1 degree each step for combined annealing/extension, wait for 90 sec premelt, then wait for 5 sec afterwards.

2.7. Plane of division

Cells were stained with DAPI, and all cells undergoing mitosis along the length of the ventricle were imaged and measured for their angles of division. Percentages of cells undergoing the 3 different angles, corresponding to different modes of division, were calculated, and an unpaired two-tailed Student's T-test was performed to determine statistical significance.

2.8. Behavior Studies

All behavioral experiments were performed following the University of Ottawa's Behavioral Core facility, Animal Care and Ethics committee protocols and adhere to the guidelines of the Canadian Council on Animal Care. Age-matched DKO and control

Antibody	Dilution	Source
	Primary	
Ki67 (rabbit)	1:500	Cell Marque, SP6
Tbr2 (rat)	1:250	eBioscience
Sox2 (goat)	1:500	Santa Cruz sc-17320
Dcx (goat)	1:500	Santa Cruz sc-8066
Dcx (guinea pig)	1:2000	Millipore
Mash-1 (mouse)	1:200	BD Biosciences
E2F3a (mouse)	1:100	NeoMarkers MS-1063-P1
E2f3 (rabbit)	1:1000	Santa Cruz (sc- 878)
Actin (mouse)	1:10 000	Sigma
	Secondary	
Alexa Fluor-488	1:500	Molecular Probes
Cy3	1:500	Jackson Immuno
Dylight -649	1:500	Jackson Immuno

Table 2. Antibodies used for Immunohistochemistry and Western Blotting

mice were maintained on a mixed FVBN/C57/S129 background.

2.8.1. Fear Conditioning

Fear conditioning experiments, used to test hippocampus- and amygdala-associated learning and memory, were performed according to (Villasana et al., 2010) with some alterations. On the first day of the experiment, referred to as training day, DKO and control animals were placed into an Ethovision PhenoTyper box (Noldus Information Technology, North America) for 2 minutes, then were subjected to a 30 second, 90 dB tone coupled with a 2 second, 0.31 mA foot-shock delivered twice with a 1 minute inter-stimulus interval.

During the second day, also known as context day, the mice were placed back in the same fear conditioning setting, they were placed in on the previous day while maintaining noise and smell constant by using the same detergent, for six minutes, in order to perform the contextually conditioned fear testing without administering any tone or shock.

On cue day, which is the third day of the trial, mice were placed in a new context where the amount of freezing was measured during the first three minutes as the mice were left to explore the new environment. This new context consisted of a different box than the one they were placed in during training and context days, with a different set-up: the shape of the box was changed into a triangular one, with a different colored background and flooring, a different background noise, a new scent was added to the apparatus as well as different light settings. In addition to that, the mice were handled differently while being transferred into the apparatus on that day. Moreover, freezing

time occurring during the last three minutes of the experiment was measured, wherein the same cue tone as the one paired with the foot-shock on day 1 was played.

During the three days of testing, the duration of mouse immobility, or freezing time was recorded using Ethovision 11.5 XT video tracking system (Noldus Information Technology, North America), as previously described (Pham et al., 2009). Statistical analysis was performed using Excel, where percent-freezing time was calculated and normalized to baseline freezing, referring to the first 2 minutes of day-1 (pre-tone). An unpaired two-tailed Student's T-test was performed to assess statistical differences between both genotypes.

Prior to this experiment, foot-shock sensitivity analysis was performed as previously described (Klemenhagen et al., 2006), where 5 mice were placed into a Fear Conditioning apparatus. A foot shock of 0.05mA was administered and increased gradually by 0.05mA. Each intensity was tested three times and the intensity required for each mouse to flinch, move, jump and/or vocalize was recorded. The test was stopped when two of these reactions were simultaneously recorded as a measure of the shock threshold. Shock average was calculated, and was found to be 0.31 mA for these specific mice.

2.8.2. Morris Water Maze

The Morris Water Maze is used to test for spatial learning and memory (D'Hooge and De Deyn, 2001). The first phase of this test is the training phase, where DKO and control mice were placed into a pool filled with turbid water for 60 seconds, wherein they were allowed to escape using a hidden platform placed in a specific quadrant. The pool

consists of 4 quadrants: back-right (BR), front-right (FR), front-left (FL) and back-left (BL). The mice were trained over 8 days, using visual cues on surrounding walls to guide themselves to the platform. A two-way ANOVA was performed on the means of time spent to reach the platform in both genotypes, with a minimum 95% confidence threshold to detect a difference in the mean.

Subsequently, in the second phase of the test, the mice underwent a probe day trial on the ninth day, and tested to recall the previous location of the platform that had been removed from the BR quadrant. Time to reach the platform was calculated using Ethovision Software (Noldus Information Technologies, North America) and an unpaired two-tailed Student T-test with a minimum 95% confidence threshold was performed to detect a difference in the mean. Out of 19 DKO mice and 23 controls, 3 DKO and 5 controls outliers were removed based on variation from the Standard deviation, or > 90% thigmotaxis.

RESULTS

3.1. Characterizing the requirement for activator E2Fs during developmental and adult neurogenesis

During neurogenesis, activating E2Fs have a crucial role in regulating the proliferation and maintenance of NPCs (Callaghan et al., 1999, Cooper-Kuhn et al., 2002, McClellan et al., 2007, McClellan and Slack, 2007, Julian et al., 2013). Prior unpublished data from our laboratory described dramatically reduced neurogenesis in adult mice lacking both E2Fs 1 and 3 (Figure 5). Employing an Emx1-driven Cre model, specifically targeting the dorsal telencephalon of the developing brain, E2F1/3 DKO mice were examined during adulthood at 6 weeks of age. Analysis of the SGZ of these animals revealed significantly reduced numbers of Sox2+ apical precursor cells comparable to WT littermates, reduced proliferation by Ki67, and a dramatic decrease in the number of Dcx+ newborn neurons. We also detected a severely reduced DG in these mutants, representing an unusual and novel phenotype (Figure 5A). These results were further supported by the use of another inducible Nestin-driven CreER^{T2} model, specifically targeting NPCs within the neurogenic niches. Mice in which E2F3 was deleted at age 6-weeks, on an E2F1 null background, were examined during adulthood. Quantification of cells in the SGZ of the hippocampus similarly revealed a reduction in both proliferation of cells, marked by Ki67, and Dcx+ newborn neurons. Conversely, the population of Sox2+ type B cells showed no detectable differences (Figure 5B). Thus, these findings support the hypothesis that activator E2Fs 1 and 3 play a crucial role during adult neurogenesis.

We next asked whether E2Fs 1 and 3 modulate neurogenesis in the developing brain, or if the requirement for activator E2Fs in NPC maintenance and proliferation was adult-specific. To answer this question, we examined the NPC population during three distinct stages of embryonic development, reflecting early, middle and late embryonic neurogenesis, in E2F1/3 DKO animals.

3.1.1. The role of E2Fs 1 and 3 during cerebral cortex development

Based on the above studies showing a crucial requirement for activator E2Fs in adult neurogenesis, we asked if E2Fs 1 and 3 are required for the proliferation of NPCs in the developing cerebral cortex. Knowing that activator E2Fs are regulators of the cell-cycle and have an important role in cell proliferation during neurogenesis (Cooper-Kuhn et al., 2002, McClellan et al., 2007, Julian et al., 2013), we examined the proliferation of NPCs during early cortical development. E12.5 represents an early time-point of murine embryonic neurogenesis, during which the cortical plate starts to form, following the first wave of asymmetric divisions of RGCs (Kwan et al., 2012). We used an Emx1-Cre model to target NPCs in the developing dorsal telencephalon, where Cre recombination is active during the initiation of embryonic neurogenesis around age E10.5 (Simeone et al., 1992). To confirm the efficacy of this model at E12.5, qPCR was performed on brain tissue from mouse embryos. E2F3 mRNA levels showed an approximately 700-fold decrease in E2F3$^{flox/flox}$ Emx1-Cre positive tissue as compared to Cre-negative littermate controls (Figure 6A).

Through immunohistochemical staining, we examined the proliferation of Sox2+ apical precursor cells, Tbr2+ basal progenitors, and Dcx+ immature neurons.

Quantification of Ki67+ proliferating cells was calculated as a percentage of cell number. Analysis at E12.5 revealed that, in control animals ($E2F1^{+/-}$ $E2F3^{flox/flox}$), 85% of the Sox2+ stem-like cells were simultaneously proliferating; E2F1/3 double knockout (DKO) animals ($Emx1\text{-}Cre^{+}$ $E2f1^{-/-}$ $E2F3^{flox/flox}$) did not demonstrate any significant change in proliferation. Moreover, 59% of Tbr2+ progenitor cells were Ki67+ in controls, again similar to the proportion observed in DKO animals. Lastly, only 7% of Dcx+ newborn neurons were proliferating in control animals, with no detectable differences in DKO (Figure 6B). Thus, these findings show that the percentage of proliferating NPCs was not altered by E2F1/3 deficiency, suggesting that activator E2Fs 1 and 3 are dispensable for NPC proliferation during early development.

We next asked if deregulation of E2Fs 1 and 3 would have an effect on NPC proliferation and total cell number during the peak period of embryonic cortical neurogenesis. To do this, we examined brains throughout mid-cortical development at age E15.5. During this time, most of the cortical layers have been established, and layer IV neurons are being generated (Takahashi et al., 1999). The proliferation of cells was again assessed as a percentage of Ki67 expression within each population of NPCs. Ki67+ Sox2+ cells were 81% of the apical precursor population in control animals, a number again not statistically different from the DKO animals (Figure 7A). However, while 50% of Tbr2+ cells were proliferating in control animals, only 44% were proliferating in DKO, representing a statistically significant decrease ($p=0.017$) (Figure 7B). Despite this change in the basal progenitors, the newborn neuron proliferation remained unaffected by the lack of E2Fs 1 and 3 (Figure 7C). This suggests a role for E2F1 and E2F3 in regulating proliferation of basal progenitor cells beginning at age

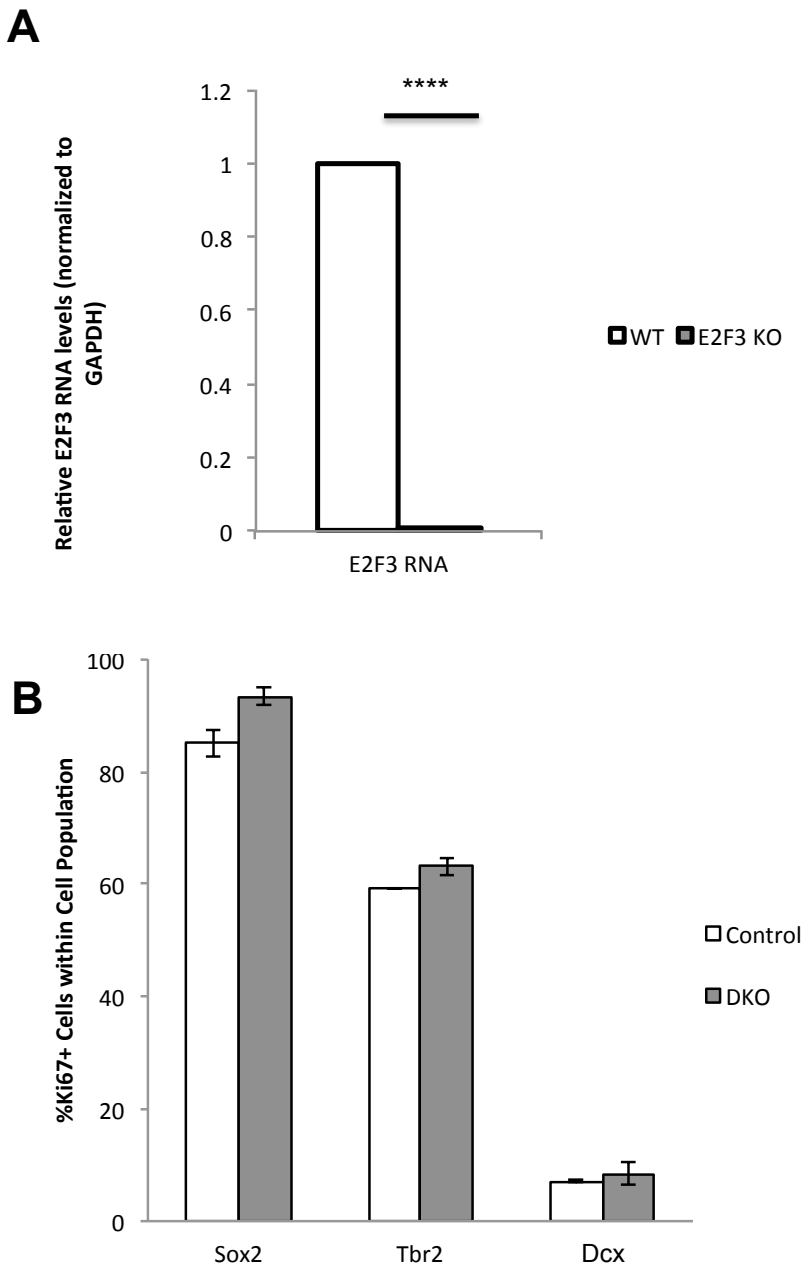

Figure 6. **No detectable change in proliferation within NPCs at E12.5 in E2F1/3 DKO using Emx1-Cre: Panel A:** Graph representing relative E2F3 mRNA levels in E2F3 Knockout (KO) (Emx1-Cre^{+} E2F3$^{flox/flox}$) brain tissue, compared to Emx1-Cre^{-} WT littermates. Values were normalized to GAPDH. Mean ± SEM, n=3 ****p<0.0001.
Panel B: Graph representing the percentage of Ki67+ cells within the Sox2 (stem-like cells) Tbr2 (progenitor cells) and Dcx (newborn neurons) in E12.5 control (E2F1$^{+/-}$ E2F3$^{flox/flox}$) and E2F1/3 DKO (Emx1-Cre^{+} E2f1$^{-/-}$ E2F3$^{flox/flox}$) in the dorsal zone of the cerebral cortex. Mean ± SEM, n=3.

E15.5, a result that leads us to question the requirement for these genes in maintaining these populations.

We then asked whether this change in proliferation correlated with changes in the total number of NPCs. Cell quantification done in four fields within a consistent unit area of 40000 μm^2 for each animal revealed no change in the total NPC population in DKO animals relative to controls. On average, 117 cells were Sox2+, and this population was not affected by deletion of both activator E2Fs (Figure 7A). Likewise, the average Tbr2+ population, which lies dorsally to that of the apical precursors, was around 80 cells with and without E2Fs 1 and 3 (Figure 7B). In addition, the newborn neurons remained at approximately 20 cells despite removing E2F1 and E2F3 (Figure 7C). This suggests that, despite being involved in regulating the proliferation of basal progenitor cells, activator E2Fs might not be required for the maintenance of this cell population at E15.5, as the numbers of cells are not changed when both E2Fs 1 and 3 are deleted. Additionally, these results show that activator E2Fs are not required for the proliferation of stem-like cells and newborn neurons during mid-corticogenesis, as we observed no changes in proliferation or number of these cells in the DKOs.

To validate the deletion of E2F3 in the Emx1-Cre model, western blot analysis was performed on E15.5 $E2F3^{flox/flox}$ Emx1-Cre positive neurospheres and compared to a Cre-negative littermate control. The results demonstrate a deletion of both E2F3a and E2F3b in the Cre+ sample, confirming gene knock-out (Figure 8A). To validate the E2F1 null mice, $E2F1^{+/+}$ and $E2F1^{-/-}$ brain tissue were extracted, and qPCR was performed. E2F1 mRNA levels exhibited a 324-fold decrease in mutants as compared to WT control (Figure 8B).

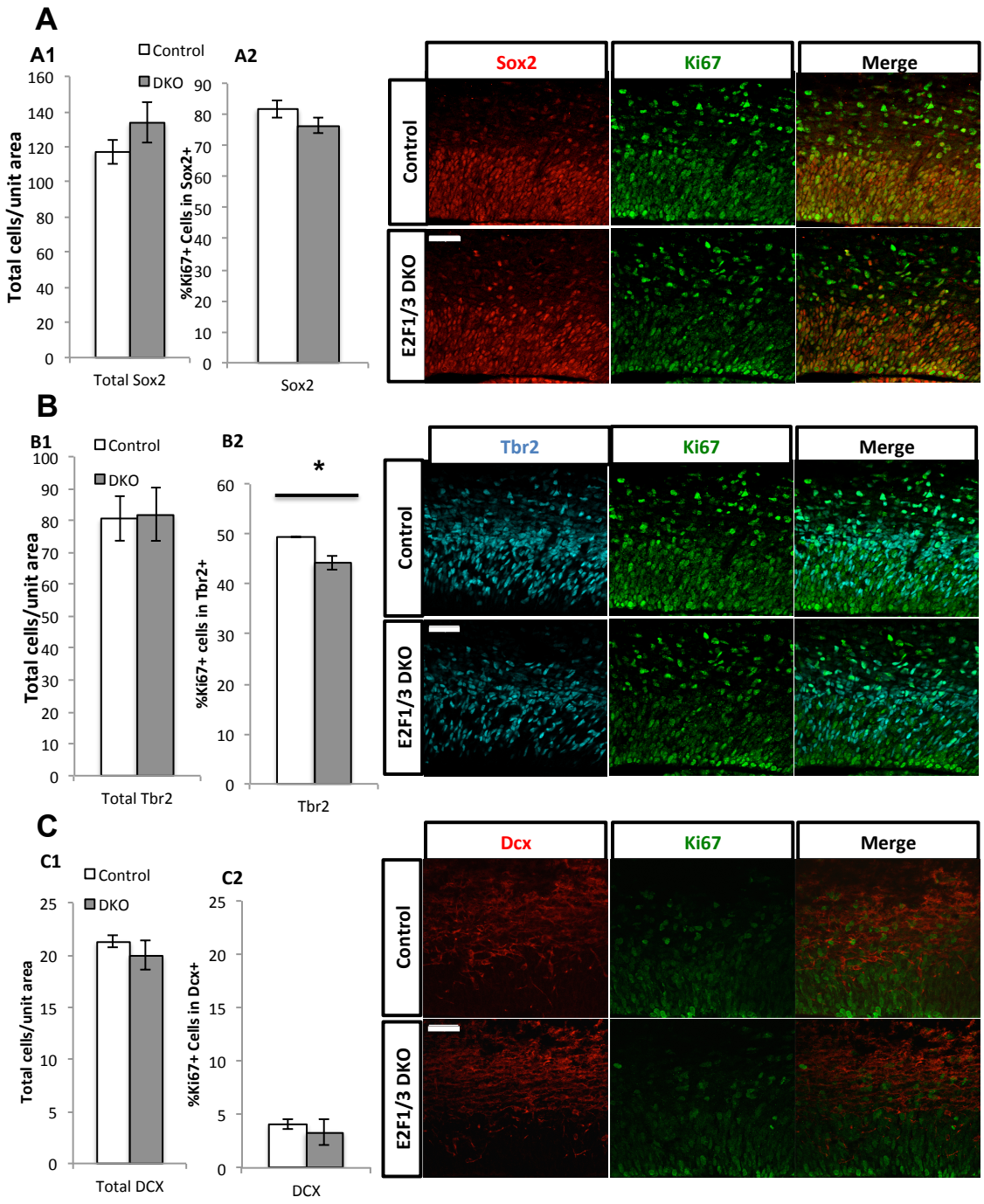

Figure 7. At E15.5, E2Fs 1 and 3 are not required to maintain NPCs in the dorsal cortex following Emx1-Cre deletion: Representative confocal images displaying (A) Sox2+ Ki67+ cells, (B) Tbr2+ Ki67+ cells and (C) Dcx+ Ki67+ cells in E15.5 control ($E2F1^{+/-}$ $E2F3^{flox/flox}$) and E2F1/3 DKO ($Emx1\text{-}Cre^{+}$ $E2f1^{-/-}$ $E2F3^{flox/flox}$) in the cerebral cortex. Graphs (A1, B1 and C1) representing the total Sox2, Tbr2 and Dcx populations per unit area (40000 μm^2) and (A2, B2 and C2) representing the % Ki67+ cells within each population. Mean ± SEM, n=3 per genotype, *$p<0.05$. Scale bar = 35 μm.

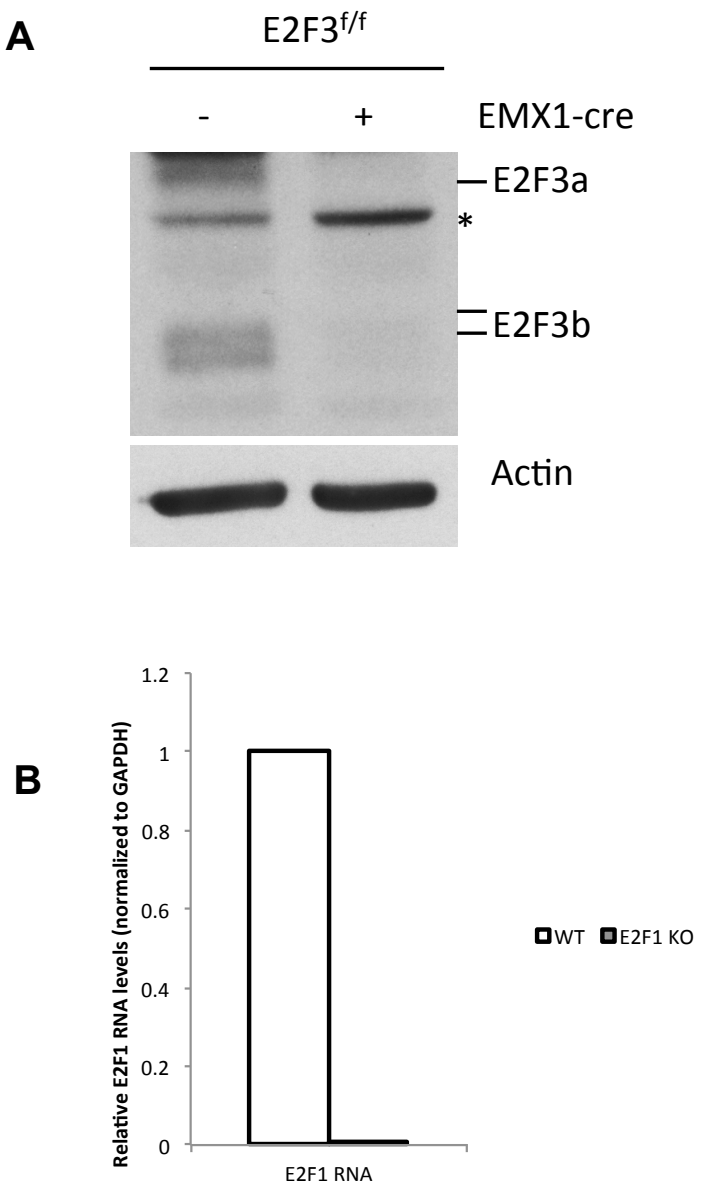

Figure 8. E2F3 and E2F1 knockout validation:

Panel A: E2F3 knockout confirmation in the Emx1-Cre model by western blot: 4-5 days Neurospheres were lysed, quantified for protein concentration and loaded onto a Western Gel which was later probed for E2F3 antibody. In the $E2F3^{flox/flox}$ Emx1-Cre^{+}, both E2F3 a and b bands are absent, which confirms knockout of the gene.*Denotes background band, based on E2F3 whole body KO which showed the same band (Julian et al., 2013).

Panel B: E2F1 Knockout validation by qPCR: Relative mRNA levels in brain tissue of E2F1 KO ($E2F1^{-/-}$) and WT ($E2F1^{+/+}$), normalized to GAPDH, showing a 324-fold decrease in the KO relative to the WT, n=1.

Previous research from our lab demonstrated that deletion of E2F3a affects the mode of division of apical precursor cells, leading to more symmetric divisions, which was consistent with *in vitro* data showing enhanced stem-cell-self renewal in knock-out animals (Julian et al., 2013). Given these findings, we asked if deletion of both E2F1 and E2F3 might affect the mode of NSC division, with potential impact on the fate of daughter cells (Godin et al., 2010, Das and Storey, 2012). A vertical plane of division (60-90°) suggests a symmetric division leading to self-renewal; whereas horizontal divisions (0-30°) and intermediate cleavage planes (30-60°) imply asymmetric divisions, generally leading to differentiation. To examine this, we measured the plane of division of DKO cells in E15.5 DAPI-stained cells undergoing mitosis. The results show no observable changes in the mode of symmetric divisions representing self-renewal, or asymmetric divisions representing differentiation, in DKO as compared to controls (Figure 9). These findings suggest no requirement for activator E2Fs in regulating the mode of division of NSCs at E15.5.

To assess the requirement for E2Fs 1 and 3 in NPCs during late development, we investigated a further time-point at E18.5, representing the end of embryonic neurogenesis following the formation of the 6 layers of the cerebral cortex (Figure 4). As we had observed a slight decrease in the proliferation of progenitor cells at E15.5 (Figure 7B), we first looked at the proliferation of NPCs. We detected a decrease in the proliferation among the apical precursor proliferation wherein the percentage of Ki67+ Sox2+ cells was decreased by half when E2Fs 1 and 3 were removed (Figure 10A). Furthermore, the percentage of proliferating cells within the Tbr2+ population was dramatically reduced by almost 3-fold, in DKO with respect to controls (Figure 10B).

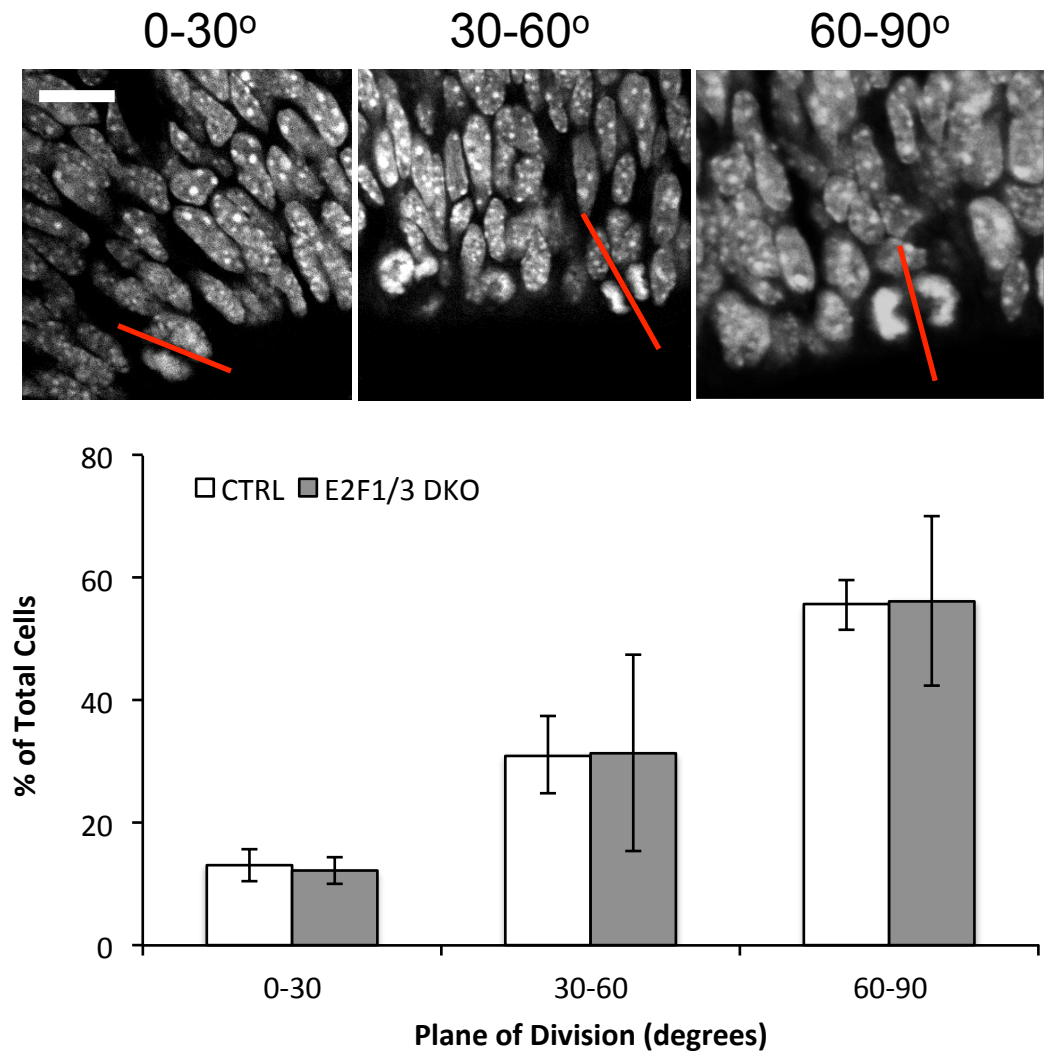

Figure 9. E2F1 and E2F3 null cells showed no detectable change in the plane of division of NSCs in the VZ of the cerebral cortex at E15.5 in Emx1-Cre mice

Top panel represents DAPI stained cells showing the three different modes of division: 0-30° represent asymmetric division, 30-60° and 60-90° represent symmetric cell division. Bottom panel represents the quantification of cells undergoing the three different modes of division, as a percentage of total cells in anaphase in E2F1/3 DKO (Emx1-Cre^{+} E2f1$^{-/-}$ E2F3$^{flox/flox}$) and control animals (E2F1$^{+/-}$ E2F3$^{flox/flox}$). Mean ± SEM, n=3 per genotype. Scale bar = 10 μm.

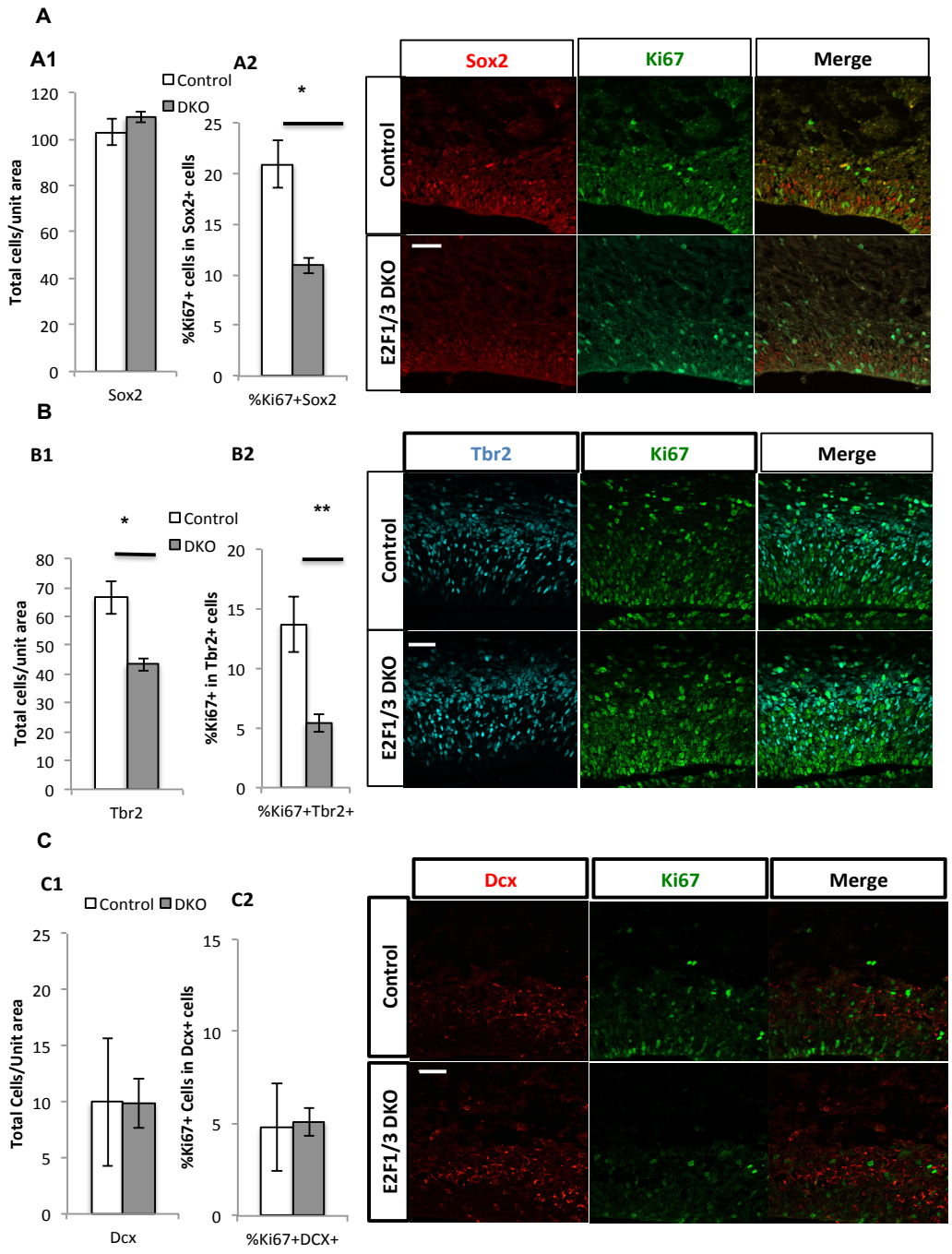

Figure 10. E2F1/3 DKO causes decrease in NPCs proliferation at E18.5 using Emx1-Cre: Immunohistochemistry against (A) Sox2 (apical precursors), (B) Tbr2 (basal progenitors), (C) Dcx (newborn neurons) and Ki67 in E18.5 control ($E2F1^{+/-}$ $E2F3^{flox/flox}$) and E2F1/3 DKO ($Emx1\text{-}Cre^{+}$ $E2f1^{-/-}$ $E2F3^{flox/flox}$) in the dorsal cortex. Graphs (A1, B1 and C1) representing the total respective cell population/unit area (40000 μm^2) and (A2, B2 and C2) representing % Ki67+ cells within each population. Mean ± SEM, n=3 per genotype, * $p<0.05$, ** $p<0.01$. Scale bar = 35 μm.

However, the Ki67+ Dcx+ population remained unchanged by this DKO (Figure 10C). We then analyzed the total cell populations to test the effect of E2Fs 1 and 3 on the number of cells present in the cerebral cortex. We noticed that while the numbers of apical precursors and newborn neurons showed no detectable changes, the Tbr2+ basal progenitor population is reduced by 1.5-fold in the DKO as compared to the controls (Figure 10B). These findings reveal that E2Fs 1 and 3 are crucial for the proliferation of apical and basal progenitor cells during late development. Moreover, the decreased number of Tbr2+ cells shows an important role for these genes in maintaining this progenitor cell population.

3.1.2. The role of E2Fs 1 and 3 in learning and memory formation

Based on our previous findings, where a profound loss of DG was detected in adult mice following embryonic loss of E2F1/3 (Figure 5), a phenotype we propose is linked to the significant reduction in NPC number, we predicted cognitive defects in mice lacking both these genes. To define the importance of E2F1 and E2F3 in the adult brain, behavioral studies were conducted employing our Emx1-Cre mice. The first study we conducted, the Morris Water Maze, was a test for spatial learning and memory (D'Hooge and De Deyn, 2001). Morris Water Maze results showed that animals lacking E2Fs 1 and 3 did not learn the location of the platform during the course of the 8 days of training (Figure 11A), while the time spent in the pool before reaching the platform decreased throughout the 8 days of the trial in control mice. In addition, DKO mice spent significantly less time, half the time that control animals completed, in the correct quadrant (BR) during probe day, suggesting that they did not recall the location of the platform (Figure 11B). In fact, DKO animals spent equal amounts of time in all quadrants

of the pool (Figure 11B). These findings show that absence of activator E2Fs 1 and 3 in the adult mouse results in defects in learning and memory formation.

To further confirm these results, a second behavioral study, the Contextual Fear Conditioning assay, was used to test hippocampus- and amygdala-associated memories (Saxe et al., 2006, Villasana et al., 2010). The results from Contextual Fear Conditioning revealed a decrease in post-tone-2 freezing time in DKO animals during day 1 (Figure 12). Moreover, there was a trend in decrease of freezing-time in both the first and last three minutes of context day, relating to the inability of DKO animals to recall the context where they received the foot shock. These findings support the hypothesis that E2F1/3 deficiency impairs learning and memory formation in adult mice. During the third day, DKO exhibited no detectable changes in the freezing time with respect to control animals, suggesting that their amygdala associated memories are not affected by E2F1/3 deletion (Figure 12).

Altogether, these findings reveal a crucial role for both E2Fs 1 and 3 in regulating the NPC population during adult neurogenesis and suggests that loss of both these genes leads to defects in learning and memory. The results further demonstrate that activator E2Fs might be dispensable during embryonic neurogenesis, as no change in NPC proliferation was observed between E12.5 and E15.5 in DKO animals. However, we propose a requirement during E18.5 and adulthood, due to an observed change in NPC proliferation at these time-points. These findings suggest the presence of a differential requirement for activator E2Fs during the different stages of neurogenesis.

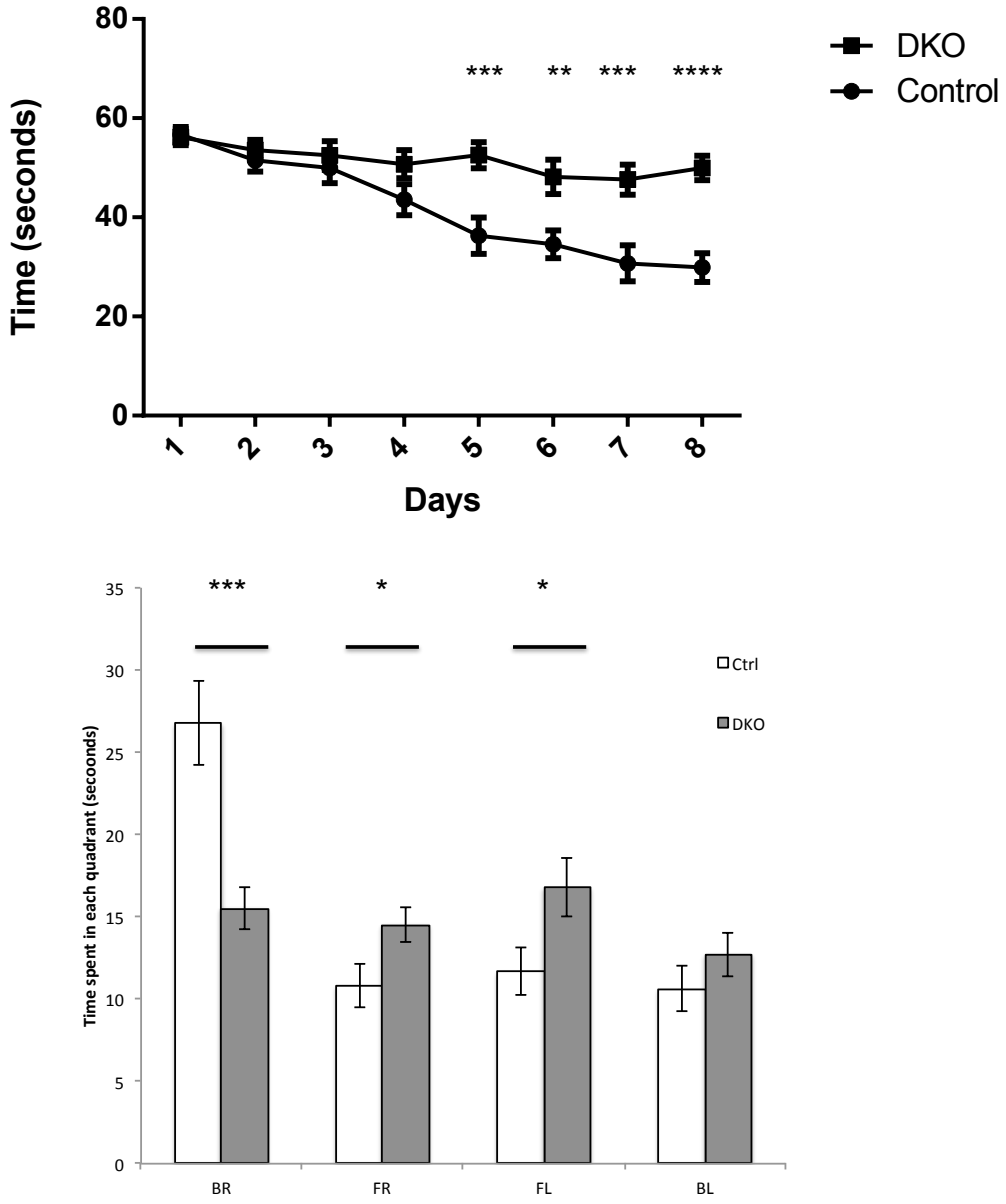

Figure 11: Loss of E2Fs 1 and 3 leads to defects in learning and memory in adult mice shown by Morris Water Maze (MWM) test:

Top Panel: MWM Training days 1-8: Graph representing the time required to escape from a pool of water within one minute during the 8 days of training, through a hidden platform using visual cues. Mean ± SEM, n=16 DKO (Emx1-Cre^{+} E2F1$^{-/-}$ E2F3$^{flox/flox}$) and n=18 controls (E2F1$^{+/-}$ E2F3$^{flox/flox}$), **p<0.01, ***p<0.001, ****p<0.0001.

Bottom Panel: MWM Probe Day: Bar graphs representing time spent in each quadrant of the pool within 60 sec of probe day trial, BR representing the quadrant where the platform used to be. Mean ± SEM, n=16 DKO (Emx1-Cre^{+} E2F1$^{-/-}$ E2F3$^{flox/flox}$) and n=18 controls (E2F1$^{+/-}$ E2F3$^{flox/flox}$), *** p<0.001, *p<0.05.

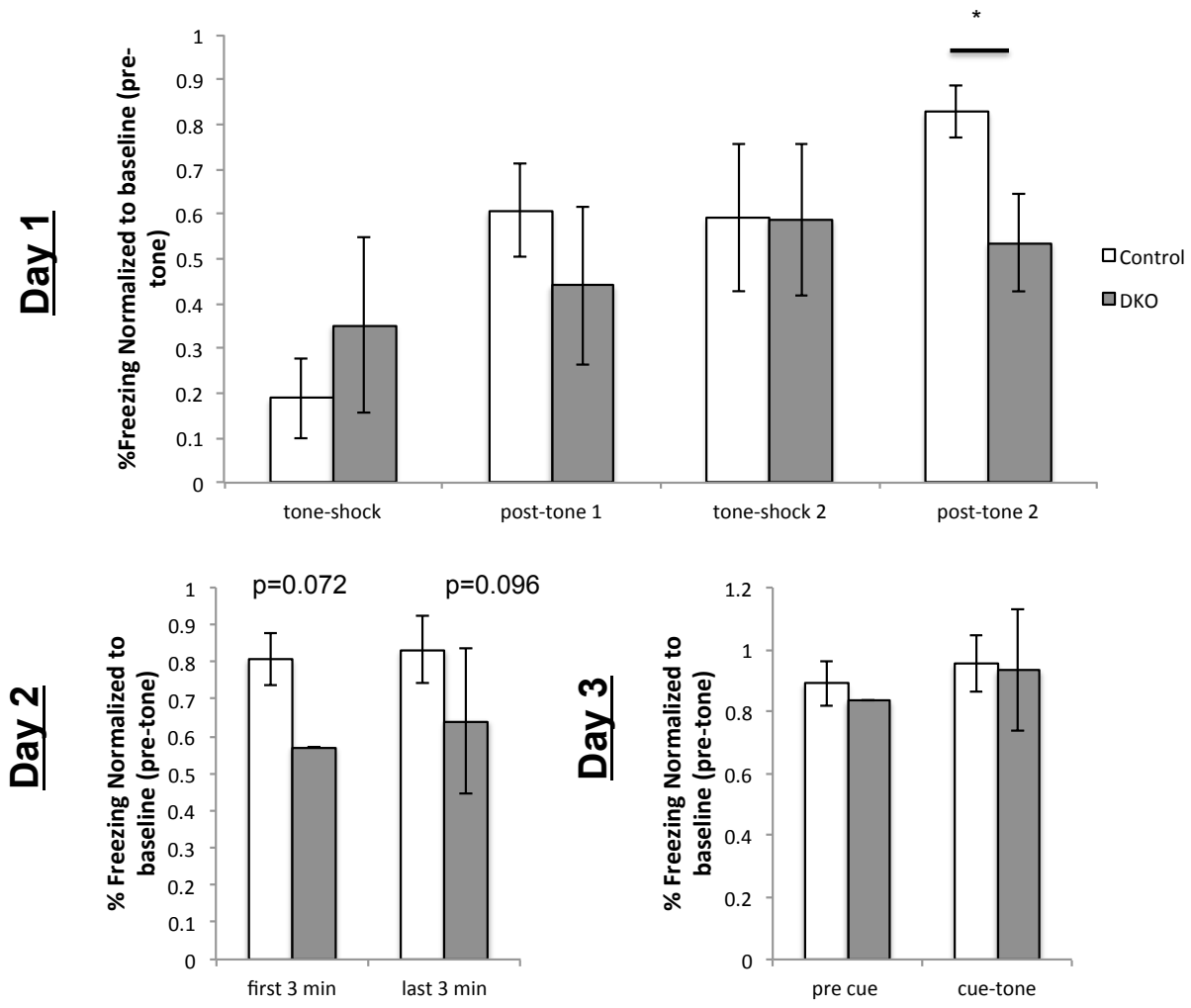

Figure 12. Decreased freezing levels in E2F1/3 DKO animals during Contextual Fear Conditioning:

Graphs representing the percentage of freezing time, normalized to baseline freezing (pre-tone). Day 1: tone-shock, post-tone-1, tone-shock-2 and post-tone 2. Day 2: first 3 min and last 3 min. Day 3: pre-cue and cue tone. Mean ± SEM, n=4 DKO (Emx1-Cre^{+} E2F1$^{-/-}$ E2F3$^{flox/flox}$), n= 7 controls (E2F1$^{+/-}$ E2F3$^{flox/flox}$), *p<0.05.

3.2. Defining the stage during which E2F requirement occurs during development

Based on the above study with the Emx1-Cre model, we concluded that absence of E2Fs 1 and 3 in the dorsal telencephalon has a dramatic effect on NPCs by E18.5, as we see a decrease in proliferation in both apical and basal precursor cells, as well as a general decrease in the basal progenitor cell population (Figure 10). This was notably not observed during early development at E12.5 or E15.5 (Figures 6 and 7). These results suggest that these genes are involved in regulating the proliferation of both apical and basal precursor cells, in addition to the size of the basal progenitor pool, during late, but not early, development. These findings lead us to hypothesize that there is a switch in activator E2F requirement for NPC proliferation and maintenance which occurs during development.

We asked when this switch in requirement occurs, and whether this shift corresponds to the transition from embryonic NPCs to adult NPCs, as these are distinct stem cell populations. To investigate the onset of this switch, we employed Nestin-driven CreER^{T2} mice, and induced E2F3 deletion, on an E2F1 null background, at specific time points (Figure 13). To confirm that NPC proliferation and neurogenesis are not disrupted by the lack of E2Fs 1 and 3 during early and mid-neurogenesis, and that the requirement for E2Fs 1 and 3 begins during late development, pregnant females were given Tamoxifen (TAM) through oral gavage at E10.5, E12.5, and E15.5 then sacrificed at E13.5, E15.5 and E18.5, respectively (Figure 13A-C). To assess whether the requirement for activator E2Fs is only neonatal, we administered TAM to E18.5 pregnant females, and sacrificed their progeny at postnatal day (P) 6 (Figure 13D). Additionally, to test the requirement for E2Fs 1 and 3 in postnatal neurogenesis, TAM was given to P21 mice over the course

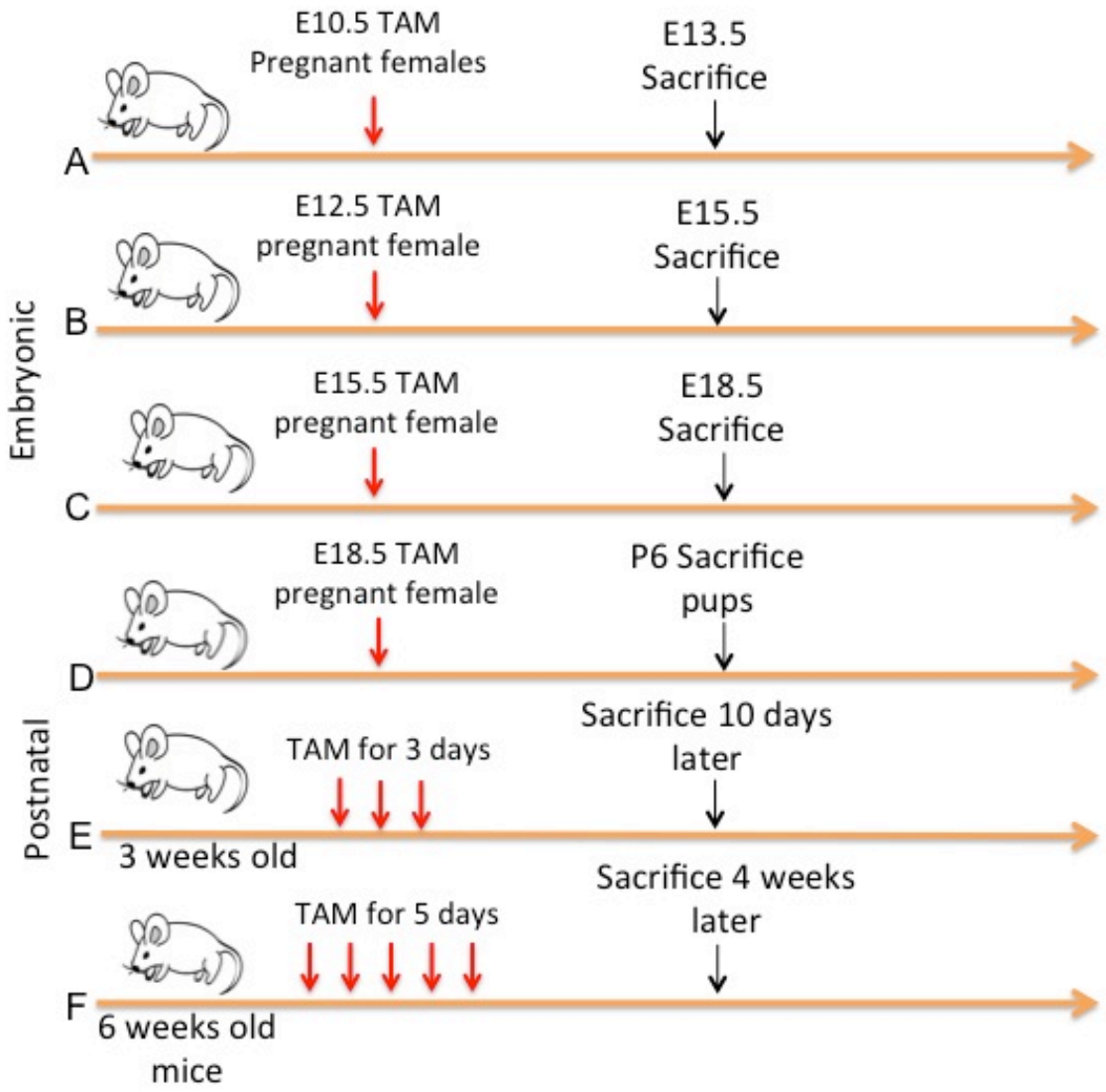

Figure 13: Time course to characterize the requirement for activator E2Fs during neurogenesis *in vivo:*
Schematic demonstrating six paradigms aimed at delineating the role of activator E2Fs in embryonic versus postnatal neurogenesis. TAM (Tamoxifen) administration is indicated by red arrows. Black arrows indicate harvesting time points, E (Embryonic day), P (Postnatal day).

of 3 days, and 6 week-old mice over the course of 5 days, and mice were perfused 10 days and 4 weeks later, respectively (Figures 13E and 13F).

3.2.1. The acute requirement for E2Fs 1 and 3 during cerebral cortex development

To validate the efficiency of the TAM-inducible Cre in recombining the E2F3 gene, qPCR was done on brain tissue from E13.5 mouse embryos that received TAM at E10.5. E2F3 mRNA levels exhibited a 13-fold decrease in $E2F3^{flox/flox}$ Nestin-$CreER^{T2+}$ tissue compared to littermate Cre-negative controls (Figure 14A). To further confirm the effectiveness of the TAM gavage in activating Cre recombination of E2F3, sections from the animals sacrificed at E15.5 were stained with E2F3a antibody and the gene deletion was confirmed (Figure 14B). To obtain E2F1/3 DKO, $E2F3^{flox/flox}$ Nestin-$CreER^{T2+}$ mice were crossed to mice on E2F1-null background, previously validated (Figure 8B). For this section, the genotype of DKO mice is Nestin-$CreER^{T2+}$ $E2f1^{-/-}$ $E2F3^{flox/flox}$, while control mice are Cre-negative, $E2F1^{+/-}$ $E2F3^{flox/flox}$.

In order to confirm the dispensability of activator E2Fs in NPC proliferation during development, a first time-point involving TAM administration at E10.5 and dissection of embryos at E13.5 was examined (Figure 13A), corresponding to early corticogenesis. Quantification of cells co-labeled with Ki67 within each precursor cell population revealed no observable changes in proliferation of NPCs in the dorsal cortex (Figure 15A). Almost 50% of Sox2+ cells were proliferating in DKO as well as littermate control animals, while 30% and 10% of Tbr2+ and Dcx+ were respectively Ki67+, in both genotypes. Furthermore, quantification of total cells of the apical and basal precursor cells, as well as the newborn neurons, revealed no detectable change in the

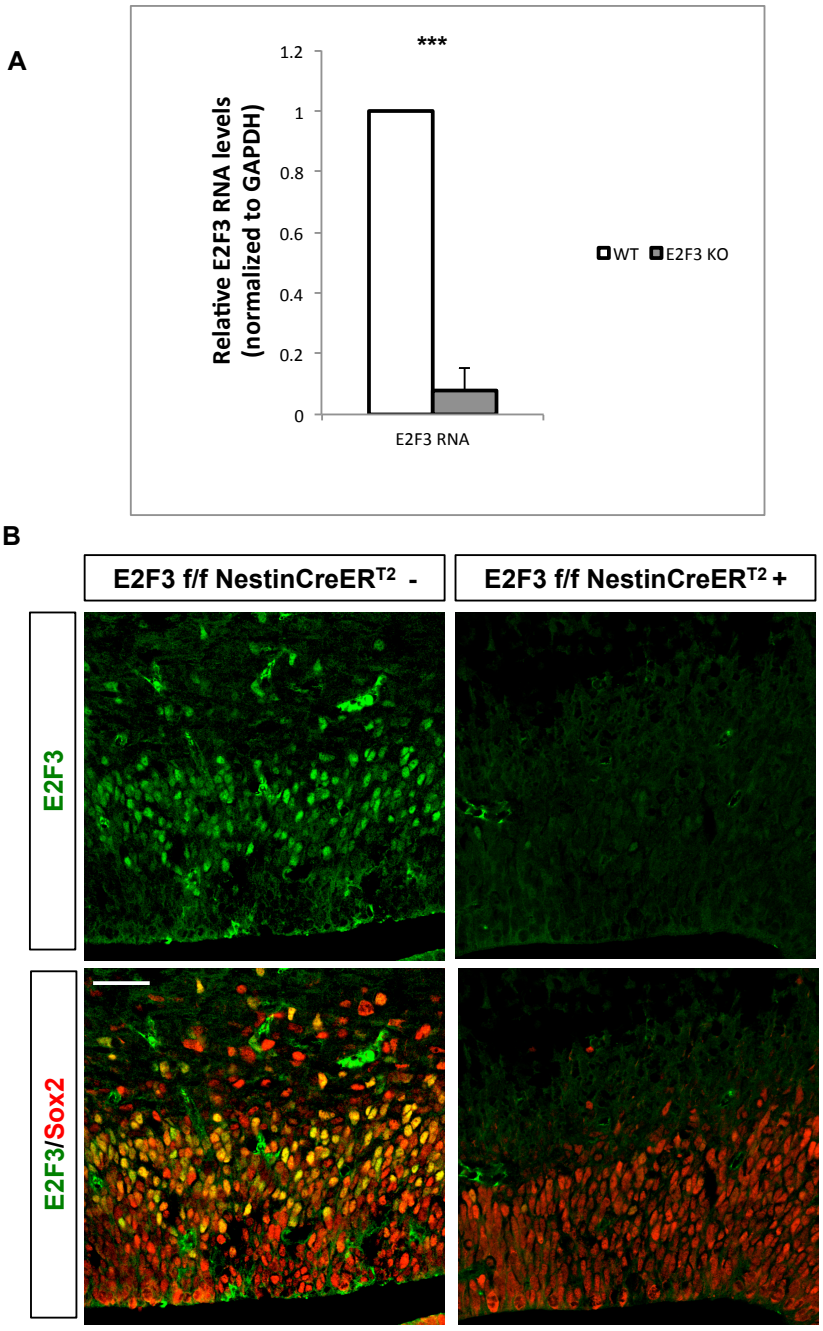

Figure 14: E2F3 knockout confirmation using Nestin-CreER^{T2} by qPCR and Immunohistochemistry: Panel A: Relative mRNA levels in E13.5 (TAM gavage at E10.5) brain tissue of E2F3 KO (Nestin-CreER^{T2+} E2F3$^{flox/flox}$) and WT control (Nestin-CreER^{T2-} E2F3$^{flox/flox}$), normalized to GAPDH. Mean ± SEM, n=3 per, ***p<0.001.
Panel B: Immunohistochemistry against E2F3a and Sox2 in E15.5 control (E2F1$^{+/-}$ E2F3$^{flox/flox}$) and E2F3 KO (Nestin-CreER^{T2+} E2F3$^{flox/flox}$) in the dorsal cortex. Some positive staining could be seen in vasculature in E2F3 KO. Pregnant mouse was given TAM at E12.5. Scale bar = 35 μm.

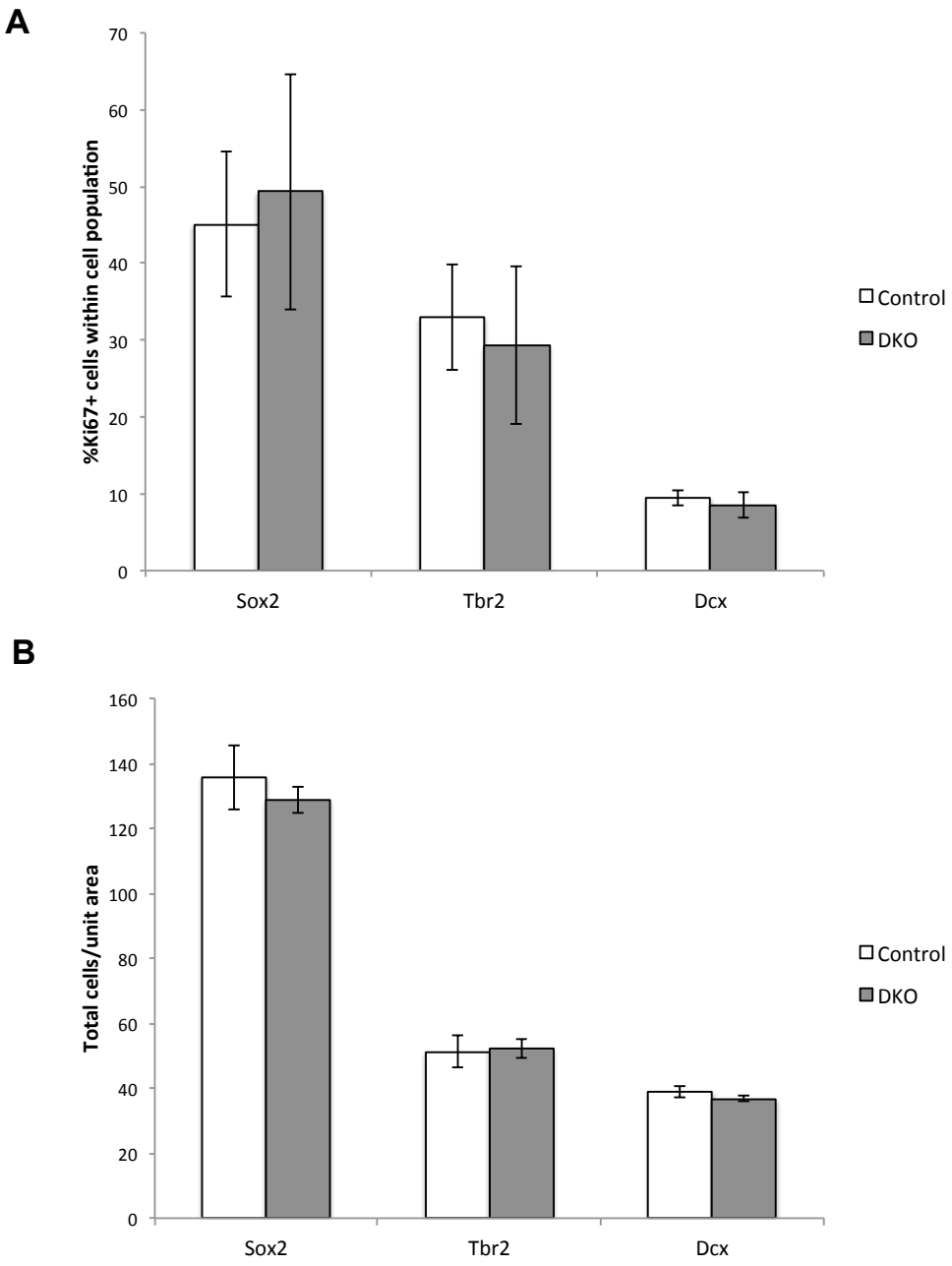

Figure 15. Deletion of E2Fs 1 and 3 shows no detectable differences in NPCs at E13.5: Animals were gavaged with Tamoxifen at E10.5, brains were dissected out at E13.5. **Panel A:** Graphs representing the percentage of Ki67+ cells within the Sox2, Tbr2 and Dcx populations in E13.5 old control ($E2F1^{+/-}$ $E2F3^{flox/flox}$) and E2F1/3 DKO (Nestin-$CreER^{T2+}$ $E2f1^{-/-}$ $E2F3^{flox/flox}$) in the dorsal cortex. Mean ± SEM, n=3, $p>0.05$.
Panel B: Graphs representing the total Sox2+, Tbr2+ and Dcx+ cells per unit area (40000 μm^2) in E13.5 old control ($E2F1^{+/-}$ $E2F3^{flox/flox}$) and E2F1/3 DKO (Nestin-$CreER^{T2+}$ $E2f1^{-/-}$ $E2F3^{flox/flox}$) in the dorsal cortex. Mean ± SEM, n=3 per genotype.

in DKO animals relative to littermate controls (Figure 15B). This data is in agreement with our previous results with the Emx1-Cre model, and suggests that E2F1 and E2F3 are not essential for maintenance and proliferation of NPCs during this early stage of embryonic neurogenesis.

To determine whether the requirement for activator E2Fs begins during mid-embryonic neurogenesis, a second time point was investigated, where Cre-recombination was induced at E12.5 and mice sacrificed 3 days later (Figure 13B). At E15.5, E2F1 and E2F3 null brains did not exhibit any difference in the number of proliferating cells in the dorsal ventricular zone of the telencephalon, supporting the hypothesis that activating E2Fs are dispensable for cell divisions during mid-cortical development. Relative to control animals, the expression of Ki67 within Sox2, Tbr2 and Dcx populations showed no detectable changes in DKO animals, and remained at 50%, 22% and 5% respectively (Figure 16 A2-B2-C2). Furthermore, total Sox2, Tbr2 and Dcx cell counts were comparable in both DKO and littermate control mice (Figure 16 A1-B1-C1), suggesting that E2F1 and 3 are dispensable for the proliferation and cell population of neural stem-like cells mid-embryonic neurogenesis.

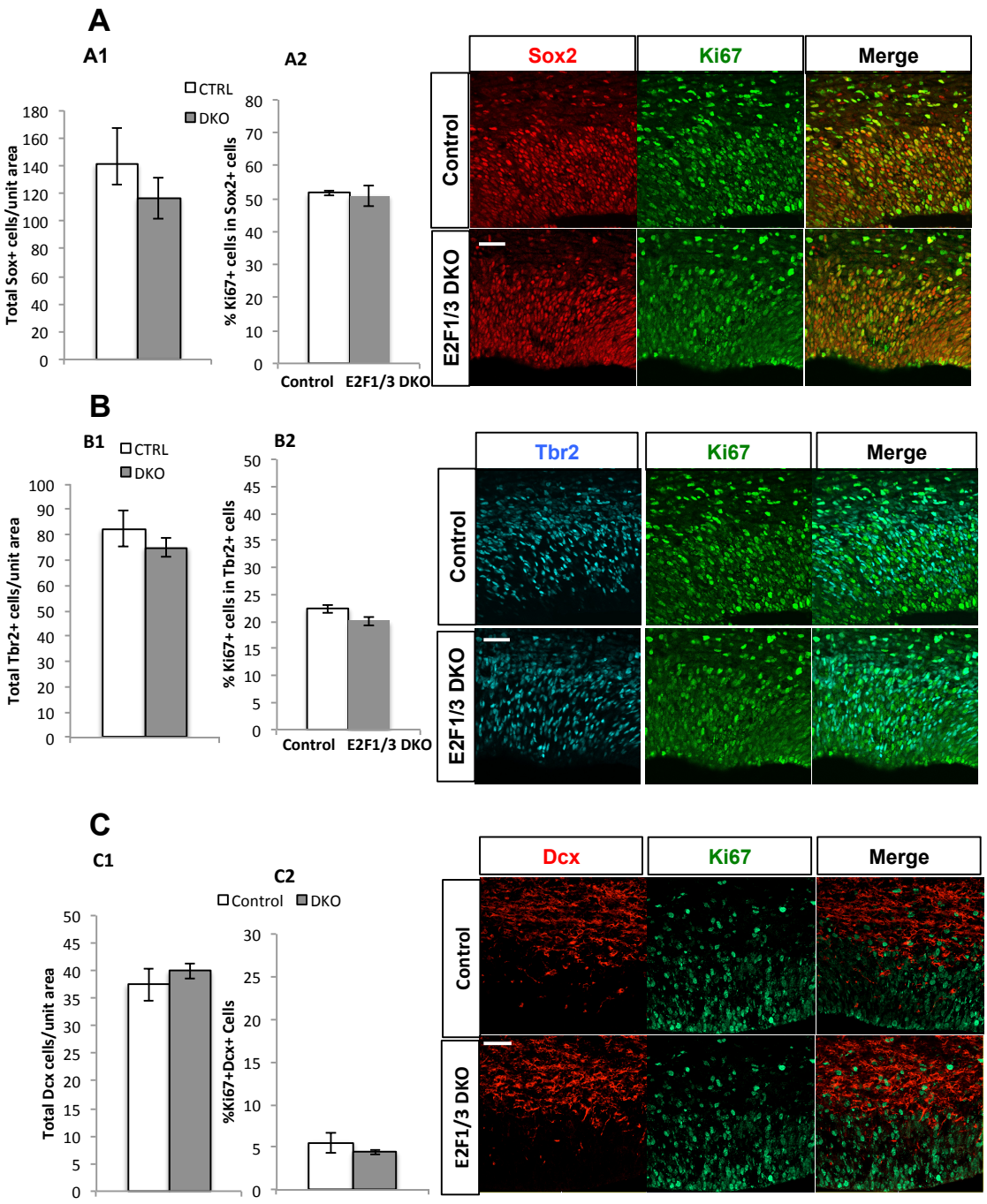

Figure 16. E2F 1 and 3 deletion shows no detectable changes in NPCs at E15.5 using Nesting-CreER^{T2}: Immunohistochemistry against (A) Sox2+, (B) Tbr2+, (C) Dcx+ and Ki67+ cells in E15.5 old control (E2F1$^{+/-}$ E2F3$^{flox/flox}$) and E2F1/3 DKO (Nestin-CreER^{T2+} E2f1$^{-/-}$ E2F3$^{flox/flox}$) in the dorsal cerebral cortex. Pregnant mice were gavaged with TAM at E12.5. Graphs (A1, B1 and C1) representing the total cell population of each of the 3 NPC types per 40000 μm^2 unit area, and graphs (A2, B2 and C2) showing %Ki67+ cells within each population. Mean ± SEM, n=3 per genotype. Scale bar = 35 μm.

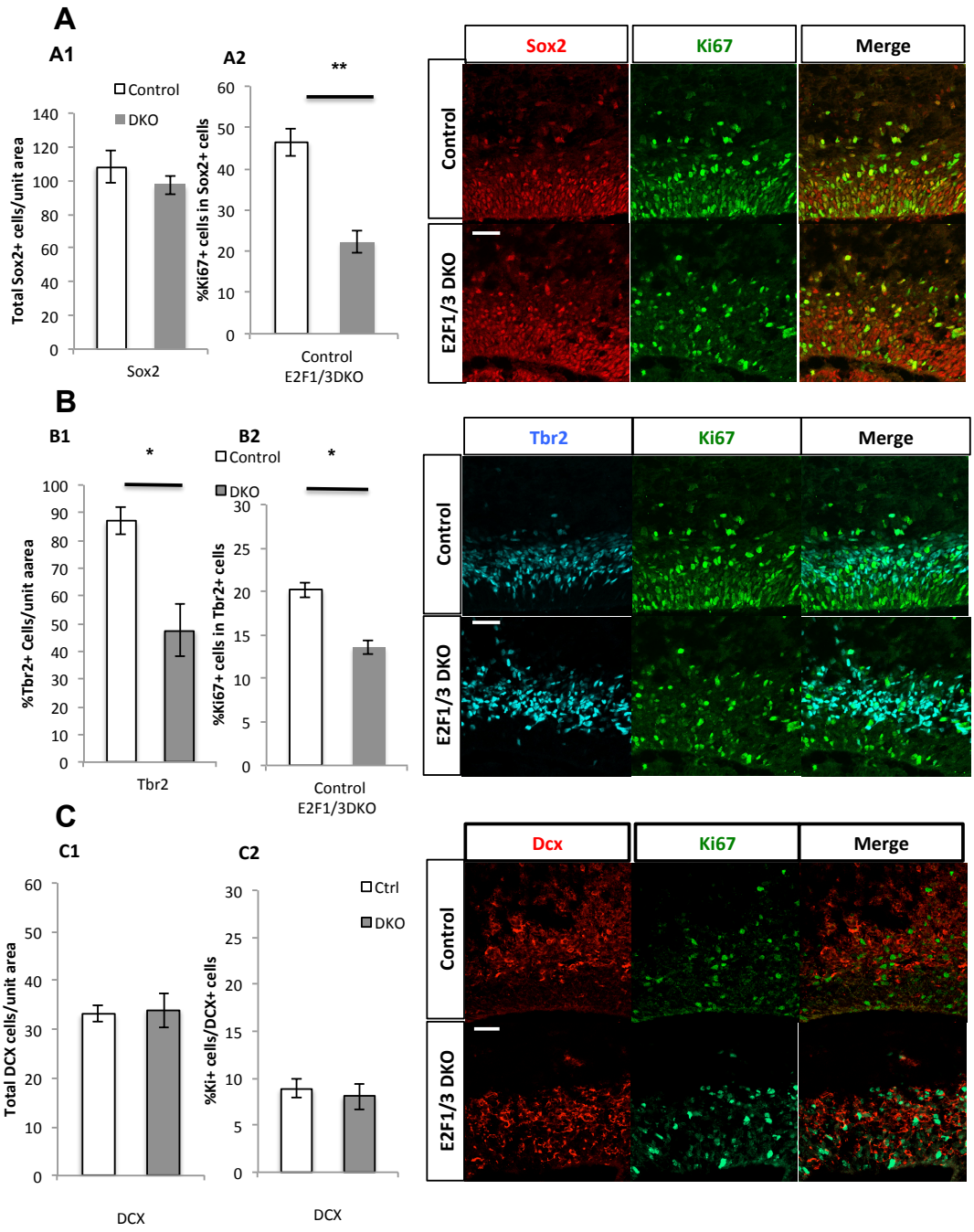

Figure 17. E2F1/3 3 DKO causes proliferation defects in NPCs at E18.5 using Nestin-CreER^{T2}: Immunohistochemistry against (A) Sox2+, (B) Tbr2+, (C) Dcx+ and Ki67+ cells in E18.5 old control (E2F1$^{+/-}$ E2F3$^{flox/flox}$) and E2F1/3 DKO (Nestin-CreER^{T2+} E2f1$^{-/-}$ E2F3$^{flox/flox}$) in the dorsal cerebral cortex. Pregnant mice were gavaged with TAM at E15.5. Graphs (A1, B1 and C1) representing the total cell population of each of the 3 NPC types per unit area (40000 μm^2), and (A2, B2 and C2) showing %Ki67+ cells within each population. Mean ± SEM, n=3 per genotype, **p<0.01 *p<0.05. Scale bar = 35 μm.

We had hypothesized, using the previous mouse model, that activator E2Fs have a switch in requirement that occurs during development. To establish the onset of this difference in E2F requirement, we administered TAM at E15.5 and then dissected pregnant female mice at E18.5 to observe embryonic brains (Figure 13C). During this late developmental time-point, the same phenotype was reproduced as seen in the Emx1-Cre model (Figure 10). The total cell population of apical precursors marked by Sox2 showed no detectable changes, however Ki67-Sox2 co-labeled cells revealed that apical precursor proliferation was decreased by half in the mutant mice relative to controls (Figure 17A). In addition, the entire basal progenitor cell population, identified by Tbr2 expression, and their proliferative capacity, were significantly reduced in mice lacking both E2Fs 1 and 3 (Figure 17B). Ki67+ Dcx+ newborn neurons, showed no observable effects with this double-gene deletion (Figure 17C).

These results demonstrate that the requirement for activator E2Fs begins at the end of embryonic neurogenesis. In addition, these data further supports the hypothesis that activator E2Fs are dispensable for early and mid-neurogenesis, since deletion of these genes using Nestin-CreER^{T2} had the same effect as long-term deletion using Emx1-Cre. This indicates that the decrease in proliferation at E18.5 is due solely to the loss of E2F1 and E2F3 function during late embryogenesis, and not a slow cumulative consequence of E2F1/3 loss starting at E10.5, concurrent with the onset of Emx1-Cre activation and embryonic neurogenesis.

3.2.2. The requirement for E2Fs 1 and 3 in postnatal neurogenesis

As the Rb/E2F pathway is largely known for its role in cell cycle regulation, in

addition to our previous work that demonstrated that E2F1-E2F3 deficiency results in a dramatic reduction in DG structure in the adult brain, as well as a decrease in neurogenesis (Figure 5), lead us to question the requirement for these genes during development. The decreased proliferation observed during late development with both Emx1-Cre and Nestin-CreER^{T2} mouse models lead us to further question whether proliferation of NPCs would be impaired in the SVZ of E2F 1 and 3 deficient postnatal mice. To address the importance of both activator E2Fs during neonatal neurogenesis, we gave pregnant females a single dose of TAM gavage at E18.5, allowed them to give birth, then sacrificed their pups at P6 (Figure 13E). Unfortunately, no DKO mice survived this transition, thus we could not analyze this time-point.

We then induced E2F3 deletion in 21-day old, Nestin-CreER^{T2+} E2F1$^{-/-}$ E2F3$^{f/f}$ mice, to answer the question of activator E2F requirement in postnatal neurogenesis. Knockout was induced using TAM gavage over three consecutive days, and the mice were perfused 10 days later, in order to examine their brain's NPC population (Figure 13E). Cell quantification in the SVZ of these animals revealed that the proliferating Sox2+ population was significantly decreased in the DKO relative to the controls, but not the total Sox2+ cell population, which only exhibited a trend in decrease (Figure 18). These results matched those previously found at the E18.5 time-point (Figures 10A and 17A). Conversely, the Mash1+ total progenitor cell population, representing type C basal progenitors, and Ki67 co-labeled proliferating cells were both decreased (Figure 18). Furthermore, there was a severe reduction observed in the number of Dcx+ immature neurons, as well as a decrease in the percentage of Dcx+ cells co-labeled with Ki67, suggesting a decrease in proliferation of these cells.

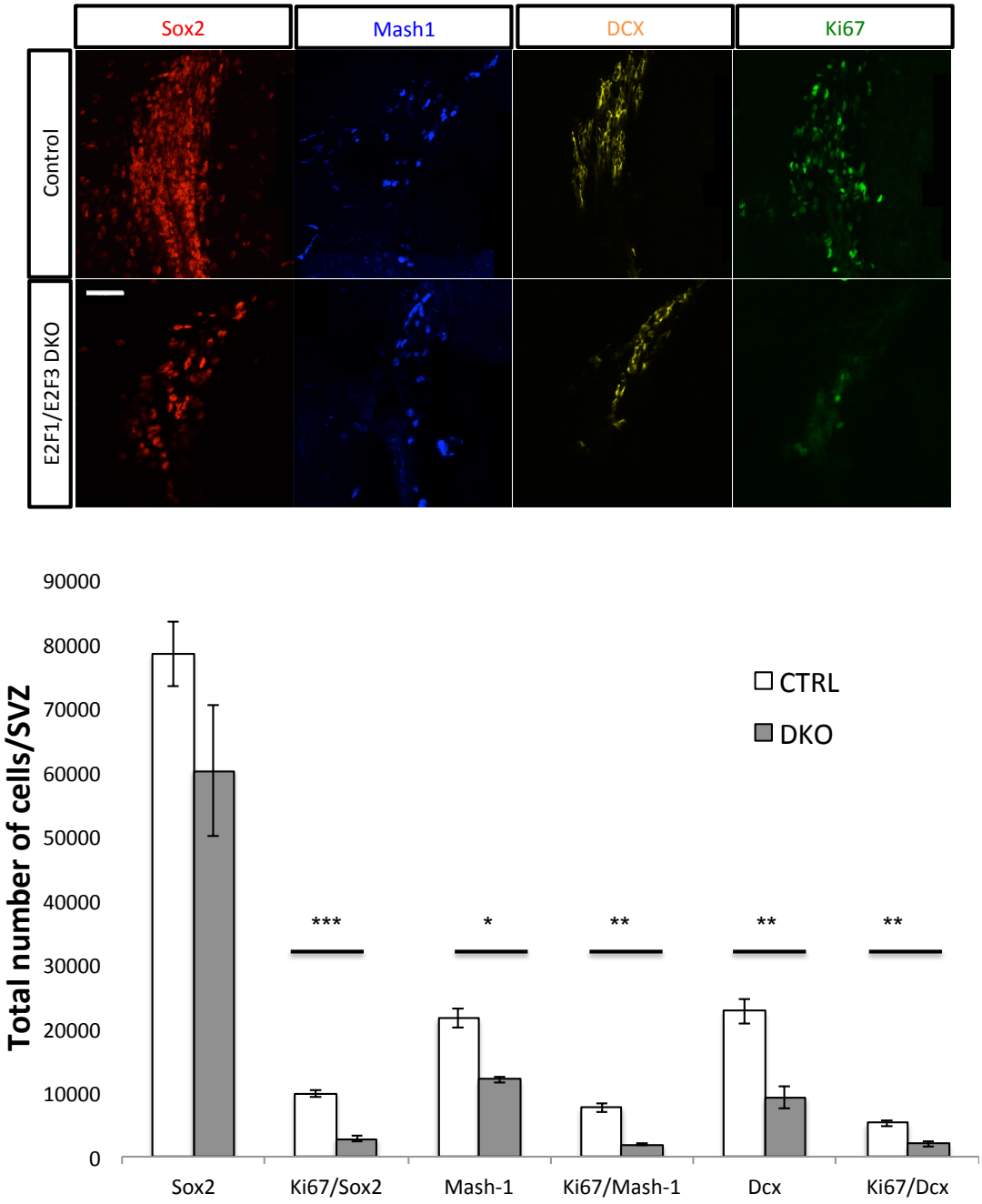

Figure 18. Population and Proliferation of NPCs are reduced in E2F1/3 DKO in postnatal day 21 mice SVZ: Representative images showing Sox2+, Mash1+, Dcx+ and Ki67+ cells in control (E2F1$^{+/-}$ E2F3$^{flox/flox}$), and E2F1/3 DKO (Nestin-CreER^{T2+} E2f1$^{-/-}$ E2F3$^{flox/flox}$) in the SVZ. TAM was given to 21-day old animals, and brains were dissected out 10 days later. Graphs representing the number of Sox2+, Mash-1+ and Dcx+ cells per SVZ, and number of Ki67+ cells within each population. Mean ± SEM, n=3 per genotype, ***p<0.001, **, *p<0.05 Scale bar = 20 μm. E. Yakubovich sectioned the tissue, performed immunohistochemistry and cell quantification.

These results reveal that absence of activator E2Fs during postnatal development leads to decreased NPC proliferation in the SVZ, and that these proteins are crucial for maintenance of neurogenesis at this stage (Figure 18).

To determine whether the disruption of activator E2Fs results in a more severe loss of NPCs in adult brains, E2F3 was deleted at age 6 weeks using 5 doses of TAM. These mice were sacrificed 4 weeks later in order to examine the effect of E2Fs 1 and 3 on adult neurogenesis in the SVZ (Figure 13F). Immunohistochemistry was performed, and cell quantification revealed a 5-fold and a 6-fold reduction in the number of proliferating cells within the Sox2+ type B cells and Dcx+ type-A cells, respectively. Moreover, the total Sox2+ population was decreased by half in the SVZ of DKO animals relative to control littermates, in addition to a 6-fold decrease exhibited in the overall Dcx+ population of mutant mice (Figure 19). This reveals a crucial requirement for activator E2Fs in regulating cell maintenance and proliferation of NPCs during adult neurogenesis, and supports our hypothesis of a differential requirement for these genes during the different stages of development. This data further supports our previous findings demonstrating a crucial role for E2Fs 1 and 3 in adult neurogenesis (Figure 5).

Taken together, these results indicate that activator E2F deficiency leads to an impairment of neurogenesis starting during late embryonic development and persisting throughout adulthood. This dramatically reduced NPC population eventually leads to defects in learning and memory formation.

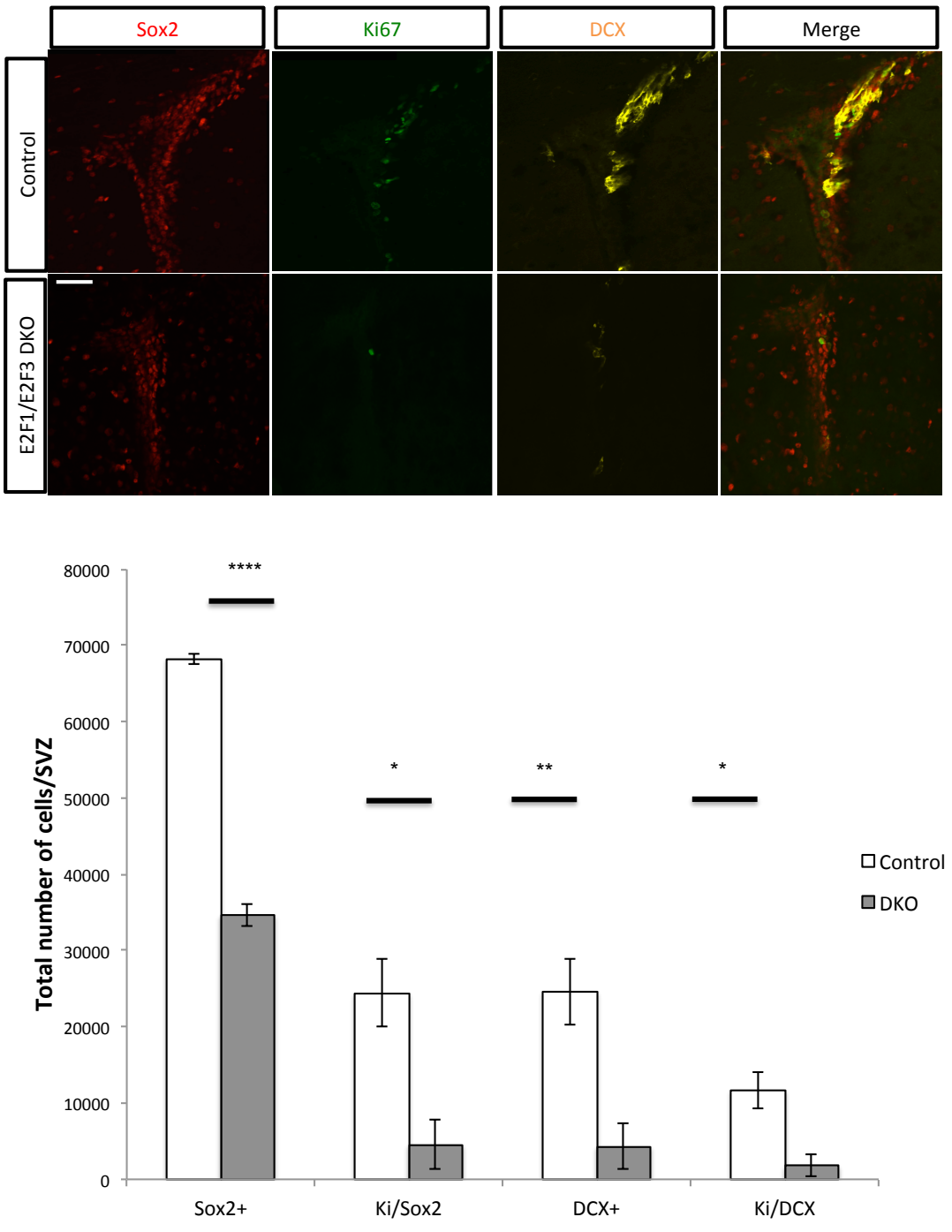

Figure 19. Population and Proliferation of NPCs are reduced in E2F1/3 DKO in adult mice SVZ: Representative images showing Sox2+, Dcx+ and Ki67+ cells in the SVZ of adult control ($E2F1^{+/-}$ $E2F3^{flox/flox}$), and E2F1/3 DKO (Nestin-$CreER^{T2+}$ $E2f1^{-/-}$ $E2F3^{flox/flox}$) animals. TAM was given to adult 6-weeks old animals, and brains were dissected out 4 weeks later. Graphs representing the number of Sox2+ and Dcx+ cells per SVZ and Ki67 co-labeled cells within each population. Mean ± SEM, n=3 per genotype, ****p<0.0001, **p<0.01, *p<0.5. Scale bar = 20 μm.

DISCUSSION

4.1. Summary of Results

In the work presented in this thesis, we have determined that activator E2Fs have different roles during NPC proliferation in embryonic and adult neurogenesis. Using a variety of time-points, activator E2Fs were shown to be dispensable early in neurogenesis, but to then adopt a crucial role in late embryonic and postnatal neurogenesis. This work provides insight regarding a novel requirement for E2Fs 1 and 3 in neurogenesis, which will be crucial in the development of future therapies designed to regulate this process. Analysis of mice null for E2Fs1 and 3 revealed three main points:

1) The requirement for activator E2Fs varies along the different stages of development
2) Activator E2Fs are essential for NPC proliferation in the postnatal brain.
3) Activator E2Fs are crucial for normal brain function, as deletion leads to reduction of NPCs, and results in cognitive defects.

Our results showed that E2F1 E2F3 DKO animals exhibited no observable changes in the population and proliferation of NPCs during early and mid-embryonic neurogenesis. The proliferation of apical progenitor cells begins decreasing at E18.5 DKOs. Interestingly, the population size and proliferation of basal progenitors are both reduced during this time-point as well. Further results reveal that lack of both activator E2Fs in the adult brain leads to memory defects, as animals have a difficulty remembering the platform location in the Morris Water Maze Test. Altogether, **this thesis serves to support the hypothesis that E2F1/3 are required for the proliferation of stem-like and**

progenitor cells, in addition to the formation and survival of newborn neurons, and that this requirement varies across the different stages of development, as discussed below.

4.2. Activator E2Fs are not required for proliferation during development

Our results have indicated different requirements for activator E2Fs during the different stages of neural development. During early corticogenesis, between E12.5 and E15.5, we observed normal proliferation of NPCs despite ablation of these cell cycle activators. In contrast to research advocating for the necessity of these genes for Rb-dependent activation of the cell cycle (Asano et al., 1996), our data indicates the presence of potential alternate pathways that regulate the transition into S-phase during this stage of development. This hypothesis is corroborated by several other studies also suggesting that, contrary to the traditional belief, proliferation can occur in the absence of all activator E2Fs in early myeloid development, retinal progenitors and intestinal stem cells (Chen et al., 2009a, Chong et al., 2009, Trikha et al., 2011, Wenzel et al., 2011). These studies show that, during development, E2Fs 1, 2 and 3 triple deficient cells undergo normal cell cycle entry. Interestingly, E2Fs 1-3 TKO lens epithelial cells did not display any abnormalities in proliferation when compared to controls, yet the ectopic DNA synthesis that was observed in these mutants could be associated with a defect in cell cycle exit (Wenzel et al., 2011).

In agreement with these findings, Cooper-Kuhn and colleagues had reported a decrease in adult neurogenesis in E2F1-null brains, however embryonic neurogenesis did not exhibit the same phenotype (Cooper-Kuhn et al., 2002). This study suggests that the

unaffected number of neurons in the developing neocortex null for E2F1 could be explained by a compensation from other activator E2Fs, that is hindered by the complexity of adult neurogenesis (Cooper-Kuhn et al., 2002), supporting a differential requirement for activator E2Fs during the different modes of neurogenesis. Furthermore, previous work from our lab has shown that telencephalon-specific Rb deletion does not affect proliferation of cortical stem-like cells in the VZ (Ferguson et al., 2002), which supports the hypothesis that E2Fs 1 and 3 are dispensable for proliferation in the developing brain. Taken together, these studies suggest that deleting activator E2Fs has no effect on proliferation during development.

4.3. Activator E2Fs are crucial for long-term NPC survival

Our results show a reduction in proliferation of both apical and basal progenitor cells at E18.5 in DKOs, as well as a decreased basal progenitor population, a phenotype which persists postnatally in P21 mice. This defect is even more pronounced then, as we see a decrease in the newborn neuron pool size and proliferation, as well as a trend in decrease in the apical precursor cell population. Furthermore, when E2Fs 1 and 3 are removed in adult-6-week-old mice, all the NPC population and their proliferation in the SVZ are severely reduced. These findings suggest two possible crucial roles for activator E2Fs: long-term cell survival, and/or regulation of differentiation. The role of activator E2Fs in cell survival was suggested by Chen and colleagues in their study showing that E2Fs 1-3 are crucial for postnatal survival of retinal progenitor cells, as TKO retinas had an increased p53 and apoptosis levels (Chen et al., 2009b). The pro-survival role of E2Fs 1-3 is also supported by another study, which showed an increase in apoptotic cells in the intestine lacking all activator E2Fs. Conversely, cell death is p53 independent in the

small intestine, suggesting that the cell death mechanism differs depending on the tissue type (Chong et al., 2009). Furthermore, Wenzel and colleagues have illustrated that proliferation doesn't require E2Fs 1-3 in the developing lens, yet the massive apoptosis exhibited by differentiated epithelial cells reveal a role for these proteins in terminal differentiation and cell survival (Wenzel et al., 2011). Thus, increased cell death following loss of E2F1/3 could explain our observation that DKO basal progenitors start decreasing at E18.5, and the phenotype becomes more dramatic postnatally, where the NPC population is reduced in the absence of activator E2Fs. Another explanation would be that due to the decreased proliferation, the cells exhibit defects in differentiation, which is discussed below.

Interestingly, it has been shown that activator E2Fs can act as repressors through an Rb-dependent manner in order to induce cell-cycle exit in differentiated cells (Chong et al., 2009). This role was later supported by the fact that deletion of all activator E2Fs results in an overexpression of their target genes *in vivo*, in addition to ectopic DNA synthesis, implying critical roles for these genes in repressing transcription and regulating proper terminal differentiation (Wenzel et al., 2011). This suggests that E2F1-3 could function as activators as well as repressors of the cell cycle. These data could translate to our findings in that in the absence of E2Fs 1 and 3, NPCs are unable to normally coordinate their cell cycle, leading them to be unable to differentiate. We propose that defective differentiation could explain why the defect in progenitor cells appears earlier than the defect in newborn neurons in our results, which have already differentiated at E18.5 and therein require more time for the defect to become evident.

The studies previously reported, together with our results suggest that E2Fs 1 and 3 are crucial for maintaining a NPC population, and that modulation of the Rb/E2F pathway could potentially lead to enhancement of neurogenesis in the adult. Though these are possible explanations for the phenotype of reduced adult NPCs in E2F1/3 DKO, a substantial amount of work is needed to determine the regulation of NPCs by the activator E2Fs.

4.4. Switch in requirement for activator E2F transcription factors in embryonic versus adult NPCs

Our results from both our germline and inducible transgenic models show that the requirement for E2Fs 1 and 3 is different depending on the stage of neural development. This could be due to the fact that these postnatal precursors become specified early during development, but remain quiescent until they are required to divide by various signals. This is in agreement with previous findings reporting that embryonic and adult NPCs are different in the way they cycle, with quiescence largely evident in the adult neurogenic regions (Calegari et al., 2005, Bragado Alonso et al., 2014, Furutachi et al., 2015). Through lineage tracing studies, Fuentealba and colleagues revealed that adult and embryonic NSCs share common progenitor cells. This study investigated the origin of NPCs in the adult brain and revealed that this population becomes specified early during development (E13.5-E15.5), but remains largely quiescent (Fuentealba et al., 2015). Recent work has further confirmed that a population of slowly dividing precursors emerges during mid-embryonic neurogenesis (Furutachi et al., 2015). This study also showed that a large fraction of type B cells in the adult SVZ originate from these embryonic progenitors that slow their cycles prior to E17.5 (Furutachi et al., 2015). As

we detect an emergence for activator E2F requirement at the same time as the transition between embryonic and adult neurogenesis is known to occur, our findings support the hypothesis that activator E2Fs are only required following the switch to adult NPCs. Interestingly, deletion of the p57 gene, which encodes a Cdk inhibitor responsible for increased cell division, hinders the emergence of NPCs in the adult brain (Furutachi et al., 2015), highlighting the importance of maintaining quiescence in the NPC pool.

In summary, the decreased proliferation in the Sox2+ and the Tbr2+ cell populations in DKOs at E18.5 is not fully understood, however more work could reveal a novel role for E2Fs 1 and 3 in maintaining quiescence and inducing proliferation of precursor cells. Studying the mechanism of change in proliferation patterns with E2F1/3 deletion between the different time-points will help increase our understanding of the feedback loops that maintain the various NPCs populations throughout embryonic and adult neurogenesis.

4.5. Alternate pathways compensating for lack of activator E2Fs

Our results, showing that proliferation normally occurs in the NPC pool during embryonic development of E2F1/3-deficient mice, hint that other pathways could be regulating cell cycle progression in the absence of these genes. Since activator E2Fs have overlapping roles, our lab investigated the possibility of E2F2 expression in the brain in DKOs, yet we found no evidence of E2F2 expression in the developing brain. E2F TKO mice showed similar NPC proliferation patterns during cortical development as E2F1/3 DKO (unpublished results). Therefore, other signaling pathways must be responsible for regulating proliferation and brain development in the absence of E2Fs 1 and 3. One

possibility is the Myc pathway, which works in parallel to the E2F/Rb pathway to control entry into the S-phase of the cell cycle (Santoni-Rugiu et al., 2000, Opavsky et al., 2007). Myc is constitutively expressed in cells undergoing the cell cycle and is capable of inducing quiescent cells to undergo replication through the activation of Cyclin E-Cdk-2 complexes. In the absence of E2Fs 1-3, Myc could act on these downstream targets of cell cycle activation in order to allow cell cycle progression (Santoni-Rugiu et al., 2000). Myc pathway genes are also expressed in E2F1-3-deficient retinas and help promote proliferation, suggesting a redundancy between these two cell-cycle regulatory pathways (Chen et al., 2009a), and highlighting the possibility that the Myc pathway could be compensating to induce proliferation in the case of activator E2Fs 1 and 3 deficiency. This could be tested by probing for Myc target genes in DKO brain tissue and comparing it to control using several methods such as western blotting or RNA sequencing.

Another possibility is that a novel Rb-dependent-E2F-independent pathway is compensating for the lack of E2Fs 1 and 3 in order to promote cell proliferation. In fact, a recent study used a mouse model with a defective Rb-allele, where a targeted mutation disrupted the interaction with E2F binding sites, leading to down-regulation of Rb-regulated target genes in MEFs (Cecchini et al., 2014). However, Cecchini and his colleagues observed similar proliferation patterns in the mutant when compared to wild-type cells *in vivo* as well as *in vitro*. Examination of this mouse model during development showed normal proliferation in most tissue when compared to littermate controls, despite displaying a decrease in Rb-regulated E2F target gene expression. These results suggest that cells retain the ability to enter and exit the cell cycle, regardless of loss of E2F repression, suggesting the presence of Rb-dependent E2F-independent

pathways for regulating proliferation/cell-cycle progression (Cecchini et al., 2014). This suggests that Rb could influence the cell cycle without E2F interactions, which could explain why cell-cycle entry and proliferation appear uninterrupted in our E2F1/3 DKO model during early and mid-development.

Another potential compensating pathway is be the Hippo signaling pathway, which induces cell proliferation by repressing its downstream targets YAP, TAZ and TEAD (Ehmer and Sage, 2016). Promoters of genes involved in regulating proliferation, such as BIRC5 and c-Myc, are targets for both the hippo and Rb/E2F pathway. Furthermore, studies in several systems have shown that both pathways share the mechanisms by which they overcome cell-cycle checkpoints (Hiemer et al., 2015). Additionally, inactivation of YAP and TEAD rescues the ectopic proliferation caused by E2F overexpression, eventually leading to cell cycle arrest. It has also been shown that there is a 60% overlap between E2F and YAP target genes in the liver (Ehmer et al., 2014). Hence, these studies suggest that the crucial role of the Hippo pathway-induced proliferation is E2F-dependent (Ehmer and Sage, 2016).

In summary, these studies reveal a number of potential pathways, related to the Rb/E2F pathway, which could have redundant roles, explaining the undetectable proliferation changes in our E2F1/3 DKO model during development.

4.6. Future Directions

The maintenance of a healthy pool of NPC throughout life is necessary, and study of the genes that activate stem cells in the brain is crucial in order to be able to use these genes as targets to activate adult neurogenesis in case of stroke or neurological disorder.

The most important result to pursue from this work would be to characterize this switch in activating E2F requirement from developmental to adult neurogenesis, and to understand its mechanism. For that RNA sequencing should be performed on DKO versus control tissue from E13.5 and E18.5, representing early and late corticogenesis. Up-regulated targets should be compared within the same time-point between different genotypes, as well as within the same genotype during different time-points. Genes with elevated expression levels relative to controls are likely targets for potential compensators for the lack of activator E2Fs. These genes could potentially be from the Myc family or the hippo-pathway as previously discussed.

Moreover, lineage tracing studies such as performed by Fuentealba and his colleages (Fuentealba, et al., 2015) using retroviral injection libraries could be conducted on E2F1/3 DKO Emx1-Cre mice in order to follow the population of NPC in the adult brain, to determine their fate in case of deletion of activator E2Fs in comparison to control cells. This experiment would allow us to know whether the adult NPC population dies in absence of both E2Fs 1 and 3 or whether this population doesn't form properly.

In vitro studies, such as neurosphere assays, could be performed using DKO and control brain tissue, in order to study the effect of E2F1/3 DKO on the stem cell population without any compensation from the surrounding tissue that occurs *in vivo*.

Rescue experiments, where we inject E2Fs 1 and 3 in DKO animals *in vivo* using postnatal electroporation, could be performed in order to determine the effect of acute restoration of the cell cycle activators on the NPC population. These mice should be investigated using immunohistochemistry, and behavior studies such as Morris Water

Maze and Fear Conditioning should be conducted in order to detect any improvement in learning and memory formation. *In vitro* rescue experiments could also be performed where E2Fs 1 and 3 can be injected to DKO neurospheres to study the cell autonomous effect of these genes on NSCs.

Other behavioral experiments should be conducted in order to test for the effect of enhancing neurogenesis with absence E2F1 and E2F3. For that, running wheel experiment could be performed using the Nestin-Cre ER^{T2} model, where the mice would have unrestricted access to a running wheel in their cage, allowing for the proliferation of NPCs, in order to potentially rescue the phenotype of decreased neurogenesis.

Furthermore, the trend in decrease in Sox2+ cells that occurs in postnatal day 21 mice should be further investigated, as the deletion of both activator E2Fs seems to be affecting the population, especially that 6-week old mice show a more severe reduction due to the longer duration of the knockout. Therefore, testing the population level at the earlier postnatal time-point and comparing it to the later time point is an interesting way to know how long the cells can survive without E2Fs 1 and 3. Cell death markers such as Caspase-3 can be used to detect whether NPCs undergo cell death or whether this decrease in cell population number and proliferation is a result of defective cell division.

Another interesting finding to investigate would be the potential correlation between the Rb/E2F pathway and p57. In fact, the dramatic reduction in neurogenesis that we see in E2F1/3 DKO adult mice is a phenotype that could be linked to Furutachi and colleagues' observations with p57 knockout mice (Furutachi et al., 2015). Deletion of p57 results in decreased BrdU-labeled cells and a reduction in NSC population. This

study also showed that p57 is responsible for maintaining the slow division of the subpopulation of embryonic NPCs that give rise to adult NSCs (Furutachi et al., 2015). Thus, it would be interesting to examine p57 in E2F1/3 DKO NSCs, which could be done through fluorescence-activated cell sorting (FACS) analysis of this specific population. A decrease in p57 expression in E2F1/3 DKO would suggest a feedback mechanism between activator E2Fs and p57 in order to maintain a pool of quiescent adult NSCs, which could be tested using RNA sequencing.

CONCLUSION

Our findings suggest that embryonic neurogenesis can in fact take place in the absence of activator E2Fs. However, the proliferation defects, which start at E18.5 in both transgenic lines, suggest that a change in the requirement for E2Fs occurs when neurogenesis switches from embryonic to adult. Furthermore, complete ablation of these cell cycle regulators in the adult brain results in loss of NPCs leading to memory defects. Based on these results, activator E2Fs have been proven to be essential in the regulation of adult NPCs and neurogenesis. Overall, the decreased numbers of cells in the postnatal brains of E2Fs 1 and 3 null mice suggests that modulation of the Rb/E2F pathway would eventually lead to enhanced neurogenesis in the adult, particularly in the context of brain injury in order to facilitate functional recovery following neurodegenerative disease.

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