The Role of Atypical E2fs in the Maintenance and Development of the Ependymal Cell Barrier

Delphie Dugal-Tessier

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Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

ABSTRACT

The discovery of neural stem cells within the adult CNS has indicated an enormous potential in facilitating neuronal regeneration after injury. Studies from our laboratory have suggested that manipulation of the Rb/E2f pathway can directly impact embryonic and adult neurogenesis, thereby having tremendous potential for neuronal regeneration therapies. Recently, two new members of the Rb/E2f pathway have been discovered, the atypical E2fs: E2f7 and E2f8. Initial studies have suggested that atypical E2fs regulate diverse processes such as cell proliferation, DNA-stress response, apoptosis, however, their importance in the brain are unknown. To analyze their function during brain development, we crossed *Nestin-Cre* mice with mice bearing a conditional *E2f7/E2f8* allele to delete both *E2f7* and *E2f8* in neural precursor cells. Whereas cortical development was largely unaffected by *E2f7/E2f8* deficiency, we observed an enlargement of the lateral ventricles occurring postnatally, a brain condition named ventriculomegaly. We then looked at the ependymal cells, which are the cells lining the wall of the lateral ventricles, to determine if these cells were affected by the absence of atypical E2fs. We found progressive denaturation of the ependymal cell layer, distortion of the ependymal motile cilia and reactive astrocytes within the layer. We identified *Gli3*, a component of the Sonic hedgehog pathway (*Shh*), as a target for E2fs, including atypical E2fs. We unravelled a novel mechanism by which atypical E2fs regulate the expression of *Gli3*, leading to up-regulation of Numb/NumbL, which in consequence destabilizes cadherins organization within the ependymal cell layer. In conclusion, our work suggests that *E2f7* and *E2f8* transcription factors play an essential role in maintaining the ependymal cell barrier.

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LIST OF ABBREVIATIONS

ABAM	Antibuitic antimycotic
AC3	Activated caspase-3
ANOVA	Analysis of Variance
ASD	Autism spectrum disorder
ATG	Start of protein translation
Bf-1	Brain factor 1
bHLH	Basic helix-loop-helix
Bmp	Bone morphogenetic protein
BP	Basal progenitor
bp	base pair
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
°C	Celsius
C57Bl/6	C57 black 6
C. elegans	*Caenorhabditis elegans*
Cdc6	Cell division cycle 6
Cdk	Cyclin dependent kinase
CdkI	Cyclin dependant kinase inhibitor
cDKO	Conditional double knock out
Celsr	Flamigo-related mouse Celsr family
ChIP	Chromatin immunoprecipitation
Chip	Microarray chip
Cip/Kip	Cdk interacting protein/kinase inhibitory protein
CNS	Central nervous system
Cre	Cyclization recombination enzyme
CSF	Cerebrospinal Fluid
C-terminal	Carboxy
cTKO	Conditional triple knock out
D. melanogaster	*Drosophila melanogaster*
DAKO	Antigen Retrieval solution
DAPI	4',6-diamidio-2-phenylindole
dB	Decibel
DBD	DNA binding domain
Dhfr	Dihydrofolate reductase
DIV	Days in vitro
DKO	double knock out
Dlx	Distaless related homologue
DG	Dentate gyrus

DNA	Deoxyribonucleic acid
DP	Differentiation-related Polypeptide proteins
dpi	Days post infection
DVL	Dishevelled
E	Embryonic day
E1A	Adenovirus early region 1A
E2f	E2 promoter binding factor
E-cadherin	Epithelial cadherin
EDTA	Ethylene diamine tetra-acetic acid
ERT2	Estrogen receptor T2
FBS	Fetal bovin serum
Fgf	Fibroblast growth factor
Flox	Flanked loxP sites
FoxJ1	Forkead box J1
FVBN	Friend virus B type susceptibility/NIH mouse
G0	Gap 0 phase
G1	Gap 1 phase
G2	Gap 2 phase
GABA	γ-aminobutyric acid
GE	Ganglionic eminence
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
Gli	GLI Family Zinc Finger
HDAC	Histone deacetylase
HEK	Human embryonic kidney
Hes-1	Hairy and enhancer of split-1 homolog
IgG	Immunoglobulin G
Hif	Hypoxia inducible factor
HPV	Human papilloma virus
HSC	Hematopoietic stem cells
Ink4	Inhibitor of cdk4
IP	Immunoprecipitation
ISH	*In situ* hybridization
kbp	Kilo-base pair
Ki67	Antigen Ki-67
Lhx6	LIM Homeobox 6
loxP	Locus of X-over P1
LxCxE	Lysine- any amino acid-Cystein- any amino acid- glutamic acid
LV	Lateral ventricle
M phase	Mitosis

_m	Meter
µ	Micro
MB	Marked box domain
MEF	ouse embryonic fibroblast
min	minutes
MOI	Multiplicity of infection
mRNA	Messenger RNA
n=	Number
N-cadherin	Neural cadherin
NE	Neuroepithelium
NEC	Neuroepithelial cell
Nkx2.1	NK2 Homeobox 1
NLS	Nuclear localization sequence
NPC	Neural precursor cell
NSC	Neural stem cell
N-terminal	amino
OB	Olfactory bulb
OGS	Ontario Graduate Scholarship
OGSST	Ontario Graduate Scholarship in Science and Technology
>p	Probability value
P	Postnatal day
P53	Tumor protein 53
p107	Retinoblastoma-like protein 1
p130	Retinoblastoma-like protein 2
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
P/S	Penicillin Streptomycin
PFA	Paraformaldehyde
pH	Potential of hydrogen
Ph-3	Phosphohistone 3
PP	Preplate
PPI	Prepulse inhibition
pRB	Retinoblastoma protein
QEII-GSST	Queen Elizabeth II. Graduate Scholarship
qRT-PCR	Quantitative real-time polymerase chain reaction
RGC	Radial glial cell
RMS	Rostral migratory stream
RNA	Ribonucleic acid
S100-beta	S100 calcium-binding protein B

S129	129S1/SvImJ
S phase	DNA synthesis
SEM	Standard error of the mean
Shh	Sonic Hedgehog
SCO	Subcommissural organ
Sox2	SRY (Sex Determining Region Y)-Box2
TSS	Transcriptional start site
Suv39h1	Suppressor of variegation 3-9 homolog 1 (Drosophila)
SV40	Simian Virus 40
SVZ	Subventricular Zone
Tbr2	T-box brain gene 2
Tk1	Thymidine kinase
Tuj1	Neuronal specification β-3-Tubulin
TUNEL	Terminal deoxynucleotidyl Transferase Biotin-dUTP (2´-deoxyuridine 5´-triphosphate) Nick End Labeling
Type-A	Neuroblast
Type-B	Primary progenitor or SVZ astrocytes B1/B2
Type-C	Transit amplifying progenitors
USV	Ultrasonic vocalization
Vegfa	Vascular endothelial growth factor A
VZ	Ventricular zone
Wnt	Wingless, integration 1
WT	Wild-type

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"Every day is a journey into the unkown"

- Ken Poirot

1. INTRODUCTION

1.1 Overview: Neurogenesis, SVZ niche and ependymal cells

It was once believed that the adult brain possesses no regenerative capacity. It was thought that neural brain plasticity was fixed after birth and any damage to the brain could not be repaired. It is now well established that, in mammals, there are two regions of the brain capable of generating new neurons throughout life: the subventricular zone (SVZ) of the lateral ventricle and the sub-granular zone of the hippocampal dentate gyrus (DG). Since the discovery of adult neurogenesis in the SVZ (Reynolds and Weiss 1992, Richards, Kilpatrick et al. 1992), and followed by the DG of the hippocampus (Gage, Coates et al. 1995, Palmer, Takahashi et al. 1997), research has led to many efforts in (1) determining how new neurons are generated and integrated into the neural circuitry, (2) understanding how genetic and molecular malfunction in neurogenesis can lead to disease and (3) characterizing endogenous neural stem cells (NSCs) and determining how they can be manipulated to repair the damaged brain.

Neurogenesis is a complex series of events that begins embryonically to build the central nervous system (CNS). In adult mammals, populations of NSCs reside in both the sub-granular zone (SGZ) of the hippocampus and in the SVZ, located along the wall of the lateral ventricle (LV). The adult neurogenic zone, also known as the SVZ neural stem cell niche, is a layer of dividing cells located along the wall of the LV, separated from the cerebral spinal fluid (CSF) by a single layer of multiciliated ependymal cells, also known as the ependymal cell barrier. The function of VZ ependymal cells is the primary focus of this thesis.

We are just beginning to understand the molecular and genetic events that regulate the ependymal cell barrier. High intracerebroventricular pressure, cerebral ischemia, hypoxia and neuroinflammation have been shown to alter the ependymal cell barrier leading to disorders such as hydrocephalus, also known as ventriculomegaly, which is characterized by increased CSF volume and enlargement of the ventricular cavities [Reviewed in (Bruni, Del Bigio et al. 1985, Sarnat 1995, Jimenez, Dominguez-Pinos et al. 2014)]. In recent years, defects in neurogenesis such as abnormal NSCs proliferation and neuronal differentiation have been shown to disrupt the ependymal cell barriers leading to neurological impairment associated with ventriculomegaly [Reviewed in (Guerra, Henzi et al. 2015)]. Ventriculomegaly is a rare but severe disorder-affecting children worldwide. Dilated lateral ventricles characterize this disorder. As being one of the only courses of action, surgical intervention is associated with neurological deficits and low prognosis outcome.

The retinoblastoma protein (pRb), a tumour suppressor, best known for its role in cell cycle regulation through direct repression of E2f transcription factors, can directly impact both embryonic and adult neurogenesis (Ferguson, Vanderluit et al. 2002, MacPherson, Sage et al. 2003, Chen, Livne-bar et al. 2004, Ferguson, McClellan et al. 2005, Chen, Opavsky et al. 2007, McClellan, Ruzhynsky et al. 2007, McClellan and Slack 2007, Chen, Pacal et al. 2009, Chong, Wenzel et al. 2009, Andrusiak, Vandenbosch et al. 2012, Ghanem, Andrusiak et al. 2012, Oshikawa, Okada et al. 2013). **The goal of this introduction is to highlight events that contribute to the establishment and maintenance of the SVZ neural stem cell niche, centred on the role of ependymal cells. Attention will then be focused on the role of the Rb/E2f regulatory pathway in**

cell cycle regulation, followed by evidence beyond cell cycle regulation, specifically in the forebrain. The overarching theme of this thesis is to investigate the atypical branch of the Rb/E2f pathway, which constitutes transcription factors, E2f7 and E2f8, and their involvement in the regulation of the ependymal cell barrier.

1.2 Mammalian neurogenesis

The cerebral cortex is the most complex structure in the brain and its proper organization depends on production and positioning of neurons in a highly coordinated manner. Neurogenesis is a tightly regulated series of events encompassing cell division, migration, differentiation and integration into neuronal circuits. The bulk of neurons are largely born during embryonic development. While neurogenesis persists in certain regions of the adult brain, neurogenesis is the foundation of CNS development. This next section is a brief overview on embryonic neurogenesis.

1.2.1 Onset and early neurogenesis

The telencephalon originates from the embryonic ectoderm germ layer, where the neural tube consists of a single layer of neuroepithelial cells (NECs). The onset of neurogenesis encompasses the transition of NECs into radial glial cells (RGCs). Known as the neural progenitor cells of the CNS, RGCs are capable of generating neurons, astrocytes and oligodendrocytes (Kriegstein and Alvarez-Buylla 2009). In mice, the onset of neurogenesis occurs around embryonic day 9-10 (E9-10). The RGCs will switch from symmetric division to asymmetric division, where they will give rise to an RGC daughter cell and a differentiating cell (Figure 1.1). At this stage of neurogenesis,

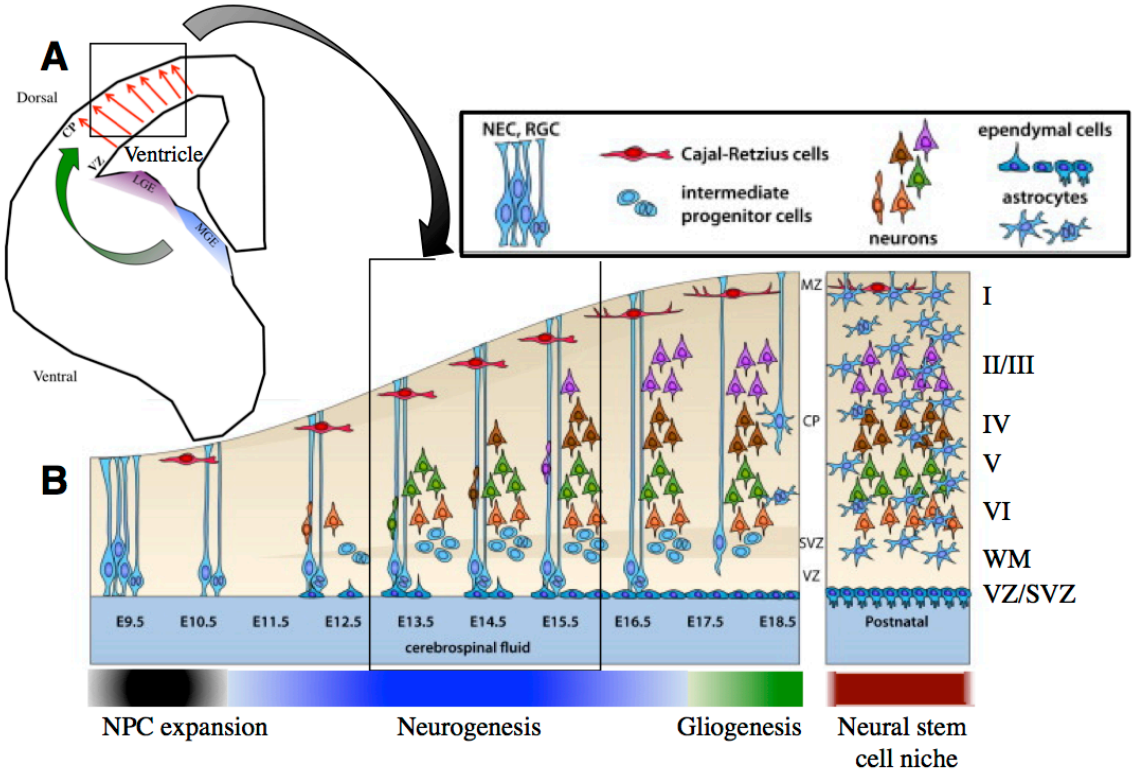


Figure 1.1: Development of the subventricular zone adult neural stem cell niche from the mouse embryonic cerebral cortex

(**A**) Illustration depicting coronal hemisection of the embryonic mouse telencephalon at E14.5. Red arrows demonstrate radial migration of cortical neurons from the ventricular zone (VZ) to the cortical plate (CP). Green arrow demonstrates tangential migration of interneurons into the lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE) to integrate into the cortex. (**B**) Illustration depicting mouse cortical development from E9.5 to the mature SVZ-neural stem cell niche. The cortex is formed in a "inside-out" manner, where the deeper layers contain early born neurons, and later born neurons will migrate past the earlier born neurons to populate the superficial layers (V, IV and III/II). The only exception is layer I, this layer is derived from the earliest born projection neurons. CP=Cortical Plate, VZ=Ventricular zone, LGE= lateral ganglionic eminence, MGE= medial ganglionic eminence, SVZ= subventricular zone, MZ= Marginal zone, WM= White matter, NEC= Neuroepithelial cell, RGC= Radial glial cells. Modified from Bjornsson et al., 2015.

this latter cell will give rise to the first neurons (Bjornsson, Apostolopoulou et al. 2015). Structurally, RGCs are highly polarized cells. Their cell bodies stretch from the apical surface of the LV to the pial (outer) surface of the cortex (Figure 1.1). The neural stem and progenitor cells, also known as neural precursor cells (NPCs), reside in the innermost apical surface of the LV also known as the ventricular zone (VZ) (Bjornsson, Apostolopoulou et al. 2015). This radial orientation of the RGCs provides scaffolding throughout the thickness of the cortex, permitting migration of NPCs from the VZ to their appropriate cortical layer. As neurogenesis progresses, RGCs will generate a basal progenitor (Gotz and Huttner 2005). These basal progenitors will under go mitosis away form the VZ and thus, forming a second germinal zone, the SVZ (Haubensak, Attardo et al. 2004, Miyata, Kawaguchi et al. 2004, Noctor, Martinez-Cerdeno et al. 2004).

1.2.2 Mid and late neurogenesis

As neurogenesis progresses, newly born neurons will migrate out of the proliferative zone and migrate to find their appropriate destination. The telencephalon cortex expands in an 'inside-out fashion', whereby earlier born neurons generate the deep cortical layers and later born neurons will migrate though the deep layers to form the superficial cortical layers (Kriegstein, Noctor et al. 2006). The mouse cortex is composed of 6 layers and two major subtypes of neurons: excitatory projection neurons and inhibitory interneurons (Kriegstein, Noctor et al. 2006). Cortical projection neurons are generated by RGCs of the dorsal cortex; while the interneurons are derived from RGCs from the ventral part of the telencephalon, know as the ganglionic eminence (GE) (Figure 1.1A) (Doetsch and Alvarez-Buylla 1996, Marin and Rubenstein 2001, Bjornsson, Apostolopoulou et al. 2015). Interneurons born in the GE will tangentially

migrate to reach the dorsal cortex and then radially migrate to reach their proper destination within specific cortical layer. As embryonic neurogenesis comes to an end, around E17-E18, basal progenitors will undergo terminal cell division to generate two daughter cells ready for differentiation (Martynoga, Drechsel et al. 2012). The end of embryonic neurogenesis triggers a switch to gliogenesis and thus, the onset of the postnatal neural stem cell niche.

1.2.3 End of neurogenesis and transition to the SVZ neural stem cell niche

As neurogenesis ends, around E18.5, RGCs begin to produce astrocytes within the postnatal SVZ. The VZ decreases in size, while the SVZ grows to support the responsibility of the mitotic active region of adult neurogenesis. During early postnatal development, the basal processes of the RGCs will retract and these cells become slow-cycling RGCs (type-B) (Merkle, Tramontin et al. 2004, Spassky, Merkle et al. 2005, Merkle, Mirzadeh et al. 2007). Postnatal RGCs will mostly generate glial cells, astrocytes and oligodendrocytes. However, a subpopulation will differentiate into ependymal cells and thus, forming the postnatal VZ. Also known as the ependymal cell barrier, these cells separate the postnatal SVZ neural stem cell niche from the CSF of the LV (Garcia-Verdugo, Doetsch et al. 1998, Merkle, Mirzadeh et al. 2007).

1.3 Establishment of the SVZ neural stem cell niche: Overview

In the adult brain, neurogenesis is present in the SVZ throughout life and is capable of producing functional neurons that integrate into neural circuits. The adult neurogenic zone is a layer of dividing cells located in the VZ-SVZ along the striatal walls of the LV (Mirzadeh, Merkle et al. 2008, Shen, Wang et al. 2008). The neural stem cell

niche consists of five main cell types: 1) ependymal cells, 2) RGCs type-B1, 3) RGCs type-B2, 4) transit amplifying progenitors (type-C) and 5) neuroblasts (type-A). Ependymal cells form a separated barrier between CSF field ventricle and the brain parenchyma. Directly adjoining ependymal cells, quiescent type-B1 cells become activated to generate a cluster of rapidly dividing type-C cells, which in turn generates dividing type-A cells (Figure 1.2) (Doetsch, Garcia-Verdugo et al. 1997, Mirzadeh, Merkle et al. 2008, Shen, Wang et al. 2008, Tavazoie, Van der Veken et al. 2008). This next section is an overview of the components of the SVZ neural stem cell niche focused on the development of the ependymal cell barrier.

1.3.1 The SVZ neural stem cell niche cytoarchitecture

The SVZ neural stem cell niche produces the largest source of newborn neurons in the mammalian brain (Gonzalez-Perez 2012). Closely associated to the ependymal side of the SVZ niche, type-B1 cells are considered the primary neural stem cells in the brain (Doetsch, Caille et al. 1999). Type-B progenitors can be subdivided into two groups based on their morphology and location within the niche: the type-B1 and type-B2 cells (Fuentealba, Obernier et al. 2012, Morrens, Van Den Broeck et al. 2012) (Figure 1.2A). Type-B1 cells retain very similar apical-basal morphology resembling the embryonic RGCs. The apical domain of the type B1 cells is tightly associated with ependymal cells through adherens and gap junctions, and can extend a process able to contact the CSF of the LV (Mirzadeh, Merkle et al. 2008). The basal domain of type-B1 cells reaches distally towards the parenchymal side of the SVZ to directly contact endothelial cells of blood vessels (Mirzadeh, Merkle et al. 2008, Shen, Wang et al. 2008).

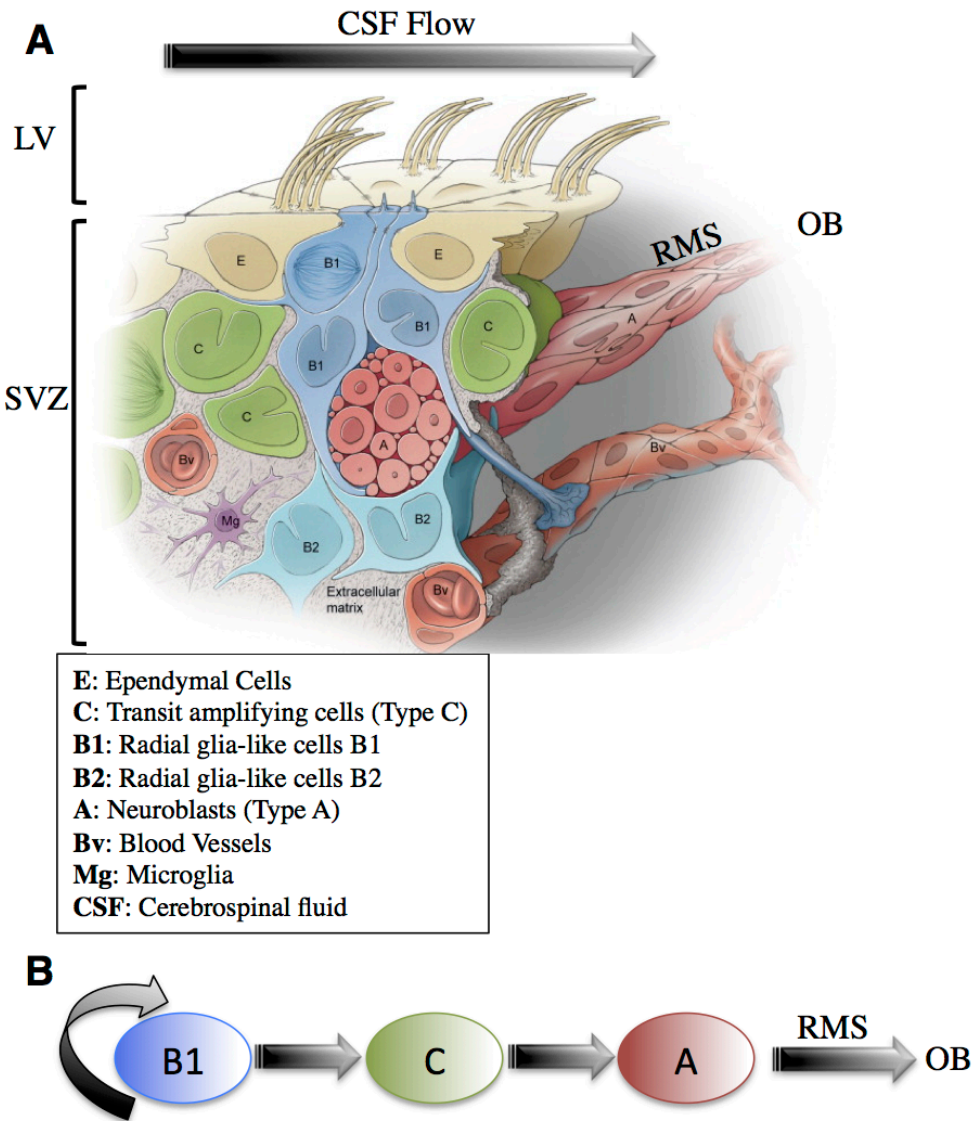


Figure 1.2: The subventricular zone adult neural stem cell niche

(**A**) Diagram depicting the subventricular zone (SVZ) stem cell niche shows, in yellow, multiciliated ependymal cells (E) lining the wall of the lateral ventricle (LV), where the cilia is oriented in the same direction as the flow of the cerebrospinal fluid (CSF). The E cells are organized in a pinwheel-like structure around the apical processes of radial glia-like B1 cells (Type B1) in blue. The basal process of Type B1 cells reaches distally to contact blood vessels (Bv) and are in close contact with transit amplifying cells (Type C), in green, and neuroblast (Type A), in red. Type A cells will migrate as a chain tangentially towards the olfactory bulb (OB), where they will terminally differentiate into neurons through the rostral migratory steam (RMS). Radial glia-like B2 cells (Type B2), in lighter blue, are located distally within the niche and contact Bv. The SVZ-neural stem cell niche also contains microglia (Mg), in purple. (**B**) Image depicting cell lineage, where the slow dividing Type B1 will divide to generate fast proliferating Type C cells, which will then generate Type A cells. The Type A cells will then migrate tangentially along the RMS to the OB. Modified from Ihrie & Alvaerez-Buylla, 2011.

Type-B2 cells are located close to the striatal parenchyma and do not have contact with the LV (Morrens, Van Den Broeck et al. 2012). It is believed that the type-B1 cells possess neurogenic capacity. Irradiation of rapidly dividing cells or use of an anti-mitotic drug show that type-B1 cells, but not ependymal cells or type-B2 cells, are capable of regenerating both type-C and A cells (Doetsch, Caille et al. 1999). The type-B1 cells are remarkably well positioned within the SVZ neural stem cell niche. The primary cilia of type-B1 cells can directly contact the CSF, through a small opening within the ependymal cell layer (Figure 1.2A). The CSF contains factors that are important modulators of NPC fate choices, such as BMPs, Wnts, Shh and retinoic acid (Huang, Liu et al. 2010, Lehtinen, Zappaterra et al. 2011). Furthermore, the intermediate domain of type-B1 cells is in direct contact with type-C and type-A cells, while the type-B1 distal foot contacts blood vessels. Blood vessels are known to support proliferation and self-renewal of SVZ NPCs (Shen, Wang et al. 2008). The apical-basal morphology of the type-B1 cells observed throughout the SVZ niche permits them to interact with multiple environments.

The quiescent type-B1 cells will divide into fast proliferating type-C cells, which give rise to type-A cells (Kriegstein and Alvarez-Buylla 2009). Type-A cells quickly leave the SVZ neural stem cell niche and migrate tangentially along the rostral migratory stream (RMS) to integrate into the olfactory bulb (OB) and differentiate into two types of interneurons: granule and periglomerular cells (Doetsch and Alvarez-Buylla 1996, Doetsch and Scharff 2001, Doetsch 2003). Although the cellular mechanisms that regulate adult SVZ NPC proliferation and self-renewal are not well known, many factors have been shown to influence this process, which includes: cell to cell communication, local vasculature and signalling input from the CSF and ependymal cells (Gonzalez-

Perez, Quinones-Hinojosa et al. 2010, Gonzalez-Perez and Alvarez-Buylla 2011, Ihrie and Alvarez-Buylla 2011).

1.3.1.1 Cell type markers of the SVZ neural stem cell niche

The type-B cells of the SVZ neural stem cell niche have astrocytic morphology and properties, and express the molecular marker glial fibrillary acidic protein (GFAP), nestin and vimentin (Figure 1.3) (Doetsch, Caille et al. 1999, Wang and Bordey 2008, Kriegstein and Alvarez-Buylla 2009, Morrens, Van Den Broeck et al. 2012). In addition, the type-B1 cells also express the cell surface marker prominin-1, also known as CD133. CD133 is a commonly used marker to identify NSCs (Coskun, Wu et al. 2008, Shmelkov, Butler et al. 2008, Beckervordersandforth, Tripathi et al. 2010). Transcription factors Dlx2 and Mash1 are markers identifying type-C cells (Doetsch, Petreanu et al. 2002, Parras, Galli et al. 2004). Doublecortin (DCX) is a common marker used to identify type-A cells (Lois and Alvarez-Buylla 1994, Rousselot, Lois et al. 1995, Francis, Koulakoff et al. 1999). Ependymal cells express the marker S100-beta (Mirzadeh, Merkle et al. 2008). Interestingly, ependymal cells also express the stem cell markers CD133 and nestin (Doetsch, Garcia-Verdugo et al. 1997, Pfenninger, Roschupkina et al. 2007, Coskun, Wu et al. 2008). Therefore, given that both ependymal cells and type-B1 cells share common markers for NSCs, it is difficult to identify NSCs purely based on markers. Many markers used to identify specific cell types in the neural stem cell niche have proven to be challenging. Many protein and transcription factors are expressed along the differentiating cells of the SVZ neural stem cell niche. For example, GFAP expression is restricted to NSCs type-B cells but is also expressed in mature astrocytes

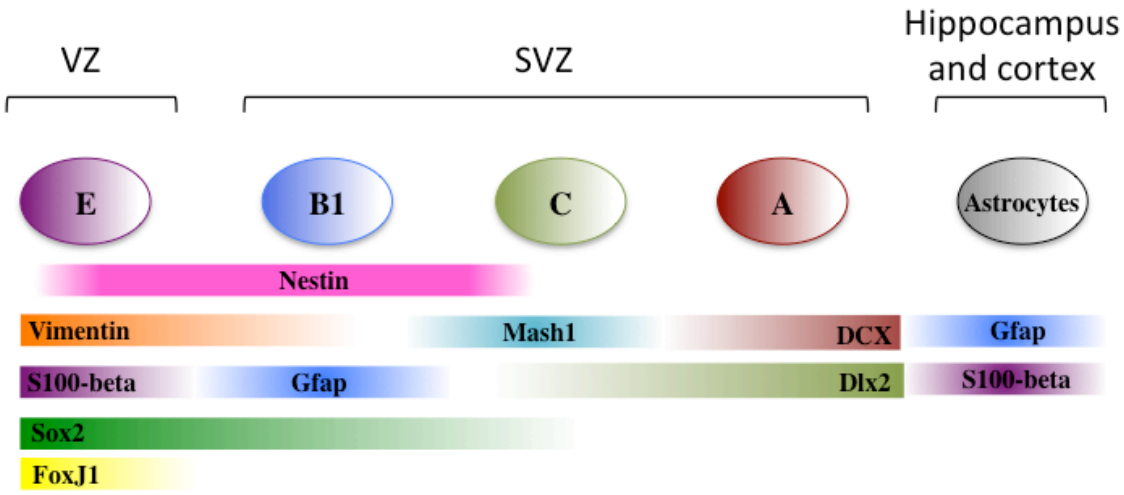


Figure 1.3: Gene expression in adult SVZ neural stem cell niche

Simplified schematic drawing is showing a model of adult SVZ neural stem cell niche and expression of markers along the cell lineage of the SVZ. Modified from Rikani et al., 2013.

(Pastrana, Cheng et al. 2009). Nestin, once believed to be a NSC specific marker, has been shown to be broadly expressed in ependymal, type-B and type-C cells (Hendrickson, Rao et al. 2011). Markers for NSCs and different cell types within the neural stem cell niche are likely to overlap; therefore, caution is required in interpreting biological characteristics of the cells comprising the SVZ neural stem niche.

1.3.2 The origin of ependymal cells

The ependyma was initially described as cuboidal and multiciliated cell barriers separating the CSF and the brain parenchyma (Bleier 1971, Millhouse 1971). It was suggested in human post-mortem studies that ependymal cell barriers was essential in propulsion of the CSF through the ventricular system (Worthington and Cathcart 1963, Cathcart and Worthington 1964). Furthermore, the ependymal cell barrier was described as being important in moving neural growth factors locally within the CSF (Roth, Kimhi et al. 1985). We now know that the multiciliated ependymal cells beat in a coordinated fashion and disruption of cilia beating is associated with disruption in the CSF flow (Brody, Yan et al. 2000, Taulman, Haycraft et al. 2001, Kobayashi, Watanabe et al. 2002, Guirao, Meunier et al. 2010, Ohata, Nakatani et al. 2014).

The immature neural tube does not possess multiciliated ependymal cells. A birthdating study shows ependymal cells are born embryonically, primarily between E14-E16 (Spassky, Merkle et al. 2005). The first ependymal cells are produced in the caudal and ventral portion of the LV and as neurogenesis progresses, ependymal cells are generated through to the rostral and dorsal axis of the LV (Spassky, Merkle et al. 2005). Immunohistochemistry and scanning electron microscopy mapping of multiciliated

ependymal cells shows no mature ependymal cells are present before birth and ependymal cell numbers drastically increase between P0 and P2 (Spassky, Merkle et al. 2005). Long-term administration of bromodeoxyuridine (BrdU) or [^{3}H] thymidine to mark mitotic cell division in mice shows labeled cells are located in the subependymal layer and are identified as type-B1 (Doetsch, Caille et al. 1999, Gregg and Weiss 2003, Spassky, Merkle et al. 2005). This study suggests that multiciliated ependymal cells are post-mitotic.

1.3.3 Postulated roles of ependymal cells in the SVZ neural stem cell niche

It was once believed that ependymal cells were the source of quiescent adult NSCs *in vivo* (Johansson, Momma et al. 1999, Wozniak 1999). By labeling and isolating cells lining the wall of the LV, it was speculated that ependymal cells were capable of generating new neurons. Since then, many studies have established the cells adjacent to the ependymal cells; type-B1 cells are the actual source of NSC in the adult brain (Chiasson, Tropepe et al. 1999, Doetsch, Caille et al. 1999, Laywell, Rakic et al. 2000). Unlike the Johansson et al., (1999) study, these other studies were able to segregate ependymal cells from the subependyma, where type-B1 cells reside. Although ependymal cells can form small spheres in cultures, they do not respond to fibroblast growth factor (FGF) and epidermal growth factor (EGF) and cannot be passaged. However, spheres from the subependyma, containing type-B1 cells, respond to growth factors and are multipotent (Chiasson, Tropepe et al. 1999).

Although the literature supports the fact that type-B1 cells are the true SVZ NSCs of the adult mammalian brain, the ability of ependymal cells to divide and self-renew is

still being debated. Some studies have shown ependymal cells may in fact exhibit stem cell characteristics in the brain after a stroke (Zhang, Zhang et al. 2007, Carlen, Meletis et al. 2009). Following cerebral ischemia, ependymal cells have been shown to acquire radial glial properties similar to type-B1 cells and could potentially generate type-A cells. However, it is not clear if it is the mature ependymal cells themselves that actually de-differentiate to generate type-A cells, or if these cells are type-B1 cells that express both radial glial and ependymal cell markers (Carlen, Meletis et al. 2009, Chojnacki, Mak et al. 2009). The confusion between both options arises from the lack of specific markers distinguishing between NSCs and ependymal cells. For example, both cell types express the stem cell marker CD133 and nestin (Pfenninger, Roschupkina et al. 2007, Coskun, Wu et al. 2008). Cell cultures sorted specifically for CD133 from the SVZ will give rise to new neurons (Pfenninger, Roschupkina et al. 2007, Coskun, Wu et al. 2008) and suggest that a sub-population of CD133-expressing ependymal could retain quiescent NSC properties. Because of the close proximity of ependymal cells and type-B1 cells, it is very difficult to distinguish between these two types of cells using NSC markers. Whether ependymal cells are capable of generating new neurons or not, research supports the fact that ependymal cells are sensitive to injury and perform important functions in the development and physiology of the brain.

1.3.4 Ependymal cell differentiation

Ependymal cells colonize the wall of the LV and undergo differentiation during the first 3 weeks of life in the mouse brain (Tramontin, Garcia-Verdugo et al. 2003, Spassky, Merkle et al. 2005). However, not much is known about the molecular

mechanism regulating ependymal cell differentiation. Determining the genetic factors separating ependymal cells from astrocyte-type cells is of particular interest in defining the formation of the SVZ neural stem cell niche.

The forkhead transcription factor, FoxJ1, is expressed postnatally in newborn ependymal cells (Jacquet, Salinas-Mondragon et al. 2009), and was one of the first factors identified as an ependymal cell differentiation regulator. FoxJ1 regulates genes associated with ependymal cell motile cilia such as γ-tubulin, dynein and kinesin motor protein and genetic ablation of *FoxJ1*, in mice, leads to complete loss of motile cilia (Jacquet, Salinas-Mondragon et al. 2009). Ependymal cells devoid of motile cilia are unable to properly differentiate. This study suggests that FoxJ1 regulates ependymal cell differentiation through genesis of motile cilia and evokes the importance of ciliogenesis in ependymal cell differentiation.

Directly after birth, RGCs differentiate into ependymal cells, a time line corresponding to the formation of the immature SVZ neural stem cell niche. Ependymal cell ciliogenesis, on the other hand, begins around P5-P8 (Spassky, Merkle et al. 2005), suggesting genetic factors other than FoxJ1 could be involved in triggering ependymal cell differentiation. Also expressed in ependymal cells, the homeobox gene *Six3* has been implicated in ependymal cell differentiation (Lavado and Oliver 2011). Conditional knockout of *Six3* specifically in nestin-expressing NPCs revealed that Six3 is important in repressing radial glial properties. In the absence of *Six3*, ependymal cells are unable to differentiate properly and retain radial glia characteristic within the wall of the lateral ventricle. This mechanism is independent of FoxJ1, since ependymal cells lacking *Six3*, still express FoxJ1 and can form multiciliated ependymal cells (Lavado and Oliver 2011).

These studies have shown two distinct mechanisms affecting ependymal cell differentiation. In the first mechanism, FoxJ1 regulates ependymal cells ciliogenesis. In the second, Six3 is required to suppress RGCs properties during ependymal cell differentiation without affecting ependymal cells characteristics. Interestingly, both these genetic models lead to dysfunction in ependymal cell differentiation and damage of the ependymal cell barrier, which ultimately leads to enlargement of the LV (Jacquet, Salinas-Mondragon et al. 2009, Lavado and Oliver 2011). Literature supports the importance of maintaining the ependymal cell barrier and disruption in the formation of this barrier can lead to ventriculomegaly. Ventriculomegaly is a brain disorder that occurs when the LV becomes dilated.

1.3.5 Arrangement of the ependymal cell barrier

The ependymal cell barrier is arranged in a tightly organized cell to cell contact environment, forming a circular 'pinwheel'-like structure, where the primary cilia of type-B1 cells reaches within the center of the pinwheel structure to interact with the CSF (Figure 1.2A). Junctional proteins, such as vimentin are located at the cell surface of the ependymal cells (Bruni, Del Bigio et al. 1985, Sarnat 1998), while adherens junctions such as calcium-dependent transmembrane adhesion molecules (cadherins), are located at the surface of ependymal cells (Lippoldt, Jansson et al. 2000, Alvarez and Teale 2007). These junctions are important in maintaining the structure of the ependymal cell barrier. Neural cadherin (N-cadherin) (Shen, Wang et al. 2008) and epithelial cadherin (E-cadherin) (Karpowicz, Willaime-Morawek et al. 2009) are expressed in ependymal cells

along the wall of the VZ and studies show that cadherins are required for maintenance of the ependymal cell barrier (Kuo, Mirzadeh et al. 2006, Rasin, Gazula et al. 2007).

1.3.5.1 Differential role of the Numb pathway in maintenance of the ependymal cell barrier

Pathways involved in embryonic NPCs differentiation are also known to be important in the function of the SVZ neural stem cell niche. For example, the Numb/Numb-like (NumbL) pathway is known to be involved in NPC maintenance and differentiation during embryonic neurogenesis (Petersen, Zou et al. 2002, Shen, Zhong et al. 2002, Li, Wang et al. 2003, Petersen, Zou et al. 2004). By antagonizing Notch signalling in one of the daughter cells, the Numb/NumbL pathway maintains the progenitor pool and differentially promote neural differentiation by regulating asymmetric cell division (Roegiers and Jan 2004). However, during the formation of the neural stem cell niche within the adult SVZ, the Numb/NumbL pathway plays two distinct roles: (1) maintenance of type-A cell survival and (2) maintenance of the ependymal cell barrier (Kuo, Mirzadeh et al. 2006).

A striking phenotype observed in a tamoxifen-induced postnatal deletion of *Numb* and *NumbL* in nestin expressing cells is a severe disruption of the ependymal cell barrier, enlargement of the LV, decrease in type-A cell survival and smaller OB, which is indicative of a decrease in adult neurogenesis (Kuo, Mirzadeh et al. 2006). To determine if type-A cell survival and maintenance of the ependymal cell barrier were regulated by one or two distinct mechanisms, Kuo et al. (2006) overexpressed the Notch pathway through the activation of intracellular Notch in the SVZ. Surprisingly, these results

phenocopied the dysfunction in type-A cell survival, thus leading to a smaller OB but without affecting the ependymal cell barrier. This study suggests the Numb/NumbL pathway regulates the maintenance of the ependymal cell barrier in a Notch-independent fashion. Interestingly, tamoxifen-induced postnatal deletion of *Numb/NumbL* shows a decrease in E-cadherin at the apical surface of the ependymal cell barrier and, furthermore, Numb can physically interact with E-cadherin (Kuo, Mirzadeh et al. 2006, Rasin, Gazula et al. 2007). Knock-down of both *N-* and *E-cadherin* can phenocopy the ependymal cell barrier disruption and thus lead to enlargement of the LV, however overexpression of both *N-* and *E-cadherin* in conjunction with a mutated form of *Numb* cannot rescue ependymal cell barrier disruption, suggesting that Numb is required for cadherin localization to the ependymal cell barrier (Rasin, Gazula et al. 2007).

1.3.5.2 Differential role of the Shh pathway in maintenance of ependymal cell barrier

Ependymal cell progenitors are born embryonically (Spassky, Merkle et al. 2005). However, abnormal neurogenesis has been linked to disruption of the ependymal cell barrier leading to ventriculomegaly (Rodriguez, Guerra et al. 2012). Little is known on the signalling events instructing embryonic RGCs to differentiate into ependymal cells. One critical signalling pathway, Sonic hedgehog (Shh), plays an essential role in neural precursor self-renewal and development of the ventral telencephalon [Reviewed in (Marti and Bovolenta 2002)]. Shh activity is mediated by the interaction of two cell surface receptors, patched (Ptch1) and smoothened (Smo). A downstream component of the

pathway, the repressor zinc finger transcription factor 3 (*Gli3*), is expressed in RGCs in the VZ but also in adult ependymal cells (Wang, Ge et al. 2011, Wang, Kane et al. 2014).

Conditional knockout of *Gli3* in nestin-expressing cells, leads to disorganization of the ependymal cell barrier and severe ventriculomegaly (Wang, Kane et al. 2014), a phenotype similar to the *Numb/NumbL* double-knockout mutants (Kuo, Mirzadeh et al. 2006). To determine the mechanism by which *Gli3* regulates the ependymal cell barrier, Wang et al. (2014) assessed a possible deregulation of the Numb/NumbL pathway. In this model, Numb was decreased followed by complete loss of E-cadherin expression at the surface of the VZ, suggesting absence of *Gli3* leads to defects in the ependymal cell barrier due to cell adhesion alteration through the Numb/NumbL pathway.

The cadherins are essential for maintaining the integrity of the ependymal cell barrier. In animal models and human cases, alteration of the ependymal cell barrier due to loss of adherens junctions such as cadherins leads to physiological abnormalities associated with ventriculomegaly [Reviewed in (Jimenez, Tome et al. 2001, Wagner, Batiz et al. 2003, Paez, Batiz et al. 2007)]. Much is still left to learn regarding the mechanisms that regulate adherens junctions and the role in maintenance of the ependymal cell barrier.

1.3.6 Ventricular wall remodelling

Adult SVZ neurogenesis is known to decline with age (Maslov, Barone et al. 2004, Luo, Daniels et al. 2006, Bouab, Paliouras et al. 2011, Capilla-Gonzalez, Gil-Perotin et al. 2012, Capilla-Gonzalez, Cebrian-Silla et al. 2013). It is believed that progressive loss of adult NPCs could contribute to the decline; however, much is still

unknown regarding other changes within the SVZ niche itself during aging, which could also affect neurogenesis. Type-A cell migration and proliferation of NPCs depends on the internal and external cues received by the niche. In young mice, approximately 2-months of age, the ependymal cell barrier is a tightly organized layer of cubical cells containing motile cilia, while in aged mice, 24-months, ependymal cells appear elongated, with ependymal cell regions devoid of cilia (Capilla-Gonzalez, Cebrian-Silla et al. 2014). In these aging mice, where cilia are present, they are mostly tangled. Proper ciliary beating is required for type-A cell migration (Sawamoto, Wichterle et al. 2006); thus, loss of cilia in aging mice could contribute to the decline in adult neurogenesis. Furthermore, ependymal cells in aged mice also express the intermediate filament GFAP (Capilla-Gonzalez, Cebrian-Silla et al. 2014). Aged mice do not generate newborn ependymal cells, supporting the hypothesis that ependymal cells are post-mitotic and do not regenerate (Spassky, Merkle et al. 2005).

Studies have shown that in aging mice, GFAP expressing cells also express the ependymal cell marker S100-beta in attempt to activate ependymal cell repair and maintenance of neurogenic capacity (Luo, Shook et al. 2008, Capilla-Gonzalez, Cebrian-Silla et al. 2014). Remodelling mechanisms and events occurring in the SVZ are poorly understood.

1.3.6.1 Reactive astrocytes and gliosis

During mild damage of the SVZ, similar to observations in the aging brain, astrocytes will incorporate within the ependymal cell layer by forming adhesion junctions with neighbouring ependymal cells (Luo, Shook et al. 2008). These astrocytes are known

to be reactive, and are the major component of the glial scar observed in the CNS following injuries. These reactive astrocytes take on the many molecular and functional characteristics similar to ependymal cells. However, it is still unknown if they can become fully functional, mature multiciliated ependymal cells. Animal models of ventricular disorders, such as hydrocephalus, most commonly display progressive loss of ependymal cells, also known as ependymal cell denudation. A model of hydrocephalus in which damage is induced by intraventricular injection of neuraminidase can induce a reactive astrocyte response (Del Carmen Gomez-Roldan, Perez-Martin et al. 2008).

High doses of neuraminidase cause severe ependymal cell denudation and obstructive hydrocephalus (Luo, Shook et al. 2008). This type of hydrocephalus is caused by accumulation of CSF within the ventricular system generally due to collapse of the aqueduct systems, channels connecting ventricles together. Low dose of neuraminidase still causes ependymal denudation; however it does not affect the aqueduct system (Luo, Shook et al. 2008). Similar to what we observe in aging mice, reactive astrocytes will integrate within the damaged ependymal cell layer. It is hypothesized that reactive astrocytes integrate to repair the damaged areas and to re-establish homeostasis between the CSF flow and the SVZ niche (Luo, Shook et al. 2008). Furthermore, through localization of E-cadherin, the *Numb*/*NumbL* and *Gli3* mice showed impairment in the ependymal cell barrier (Kuo, Mirzadeh et al. 2006, Wang, Kane et al. 2014). Analysis of these mice models showed reactive astrocytes within the ependymal cell barrier, suggesting damage and potential attempts to remodel the VZ. In cases of human ventricular disease, a layer of reactive astrocytes is also observed (Dominguez-Pinos, Paez et al. 2005, Roales-Bujan, Paez et al. 2012). Understanding the

mechanism of brain repair in response to pathological states is essential to establish therapeutic agents.

In summary, ependymal cells are now recognized as being important in the development and physiology of the adult SVZ neural stem cell niche. Our knowledge on the formation and maintenance of this multiciliated barrier is still poorly defined. Establishing the molecular mechanism regulating ependymal cell differentiation and maintenance can further our understanding of a self-repair response within the SVZ neural stem cell niche due to injury and can be useful for the design of new therapeutic techniques.

Ependymal cells are born during the peak of embryonic neurogenesis, however the mechanism regulating the switch between RGCs and ependymal cells is not well understood. The next sections will discuss the cell cycle regulator pathway Rb/E2f, a pathway shown to regulate processes important in both embryonic and adult neurogenesis. Though the role of Rb/E2f pathway in the maintenance of the ependymal cell barrier has never been assessed, it will be intriguing to determine whether components of the Rb/E2f pathway play a role in the formation of the ependymal cell barrier.

1.4 The tumour suppressor Rb/E2f pathway: Overview

The retinoblastoma tumour suppressor protein (pRb) family and E2fs are key regulators of the cell cycle machinery. In the canonical Rb/E2f pathway, the E2f transcription factors bind and activate the promoters of genes required for entry into the DNA synthesis phase (S-phase). The pRb family of proteins have the ability to arrest

cells in the gap-1 phase (G1) of the cell cycle by inhibiting the activity of the E2f transcription factors (Figure 1.4) [Reviewed in (Weinberg 1995, Harbour and Dean 2000)]. The mammalian E2f family is comprised of 8 genes that together encode for 10 different proteins (Chen, Tsai et al. 2009). In the field of cancer, initial studies concentrated on the role of pRb in cell cycle progression. We now know the Rb/E2f pathway has many roles outside of regulating G1-phase cell cycle progression, including regulation of cell proliferation, apoptosis, differentiation, senescence, DNA-damage response and repair [Reviewed in (Ren, Cam et al. 2002, Attwooll, Lazzerini Denchi et al. 2004, Dimova and Dyson 2005)]. In mammalian cells, pRb belongs to a family of pocket proteins, which includes the structurally and functionally related proteins p107 and p130 (Mulligan and Jacks 1998). The role of the Rb/E2f pathway has been a relevant question in cancer and cell cycle progression. Every year, new studies elaborate on the novel roles for the Rb/E2f pathway outside the canonical cell cycle regulation. Broadly, this thesis will further focus on an examination of the newly identified atypical branch (E2f7 and E2f8) of the Rb/E2f pathway, specifically regarding their functional role in CNS development.

1.4.1 History of the first tumour suppressor-pRb

For many years, a link between genetics and the individual predisposition to cancer has been apparent. In the early 1970s, Knudson analyzed hereditary patterns in children with the retinal cancer termed 'Retinoblastoma' (Knudson 1971). Children with a family history of retinoblastoma were more likely to develop multiple retinoblastoma tumours in one or two eyes at a very young age, while children with no history were more

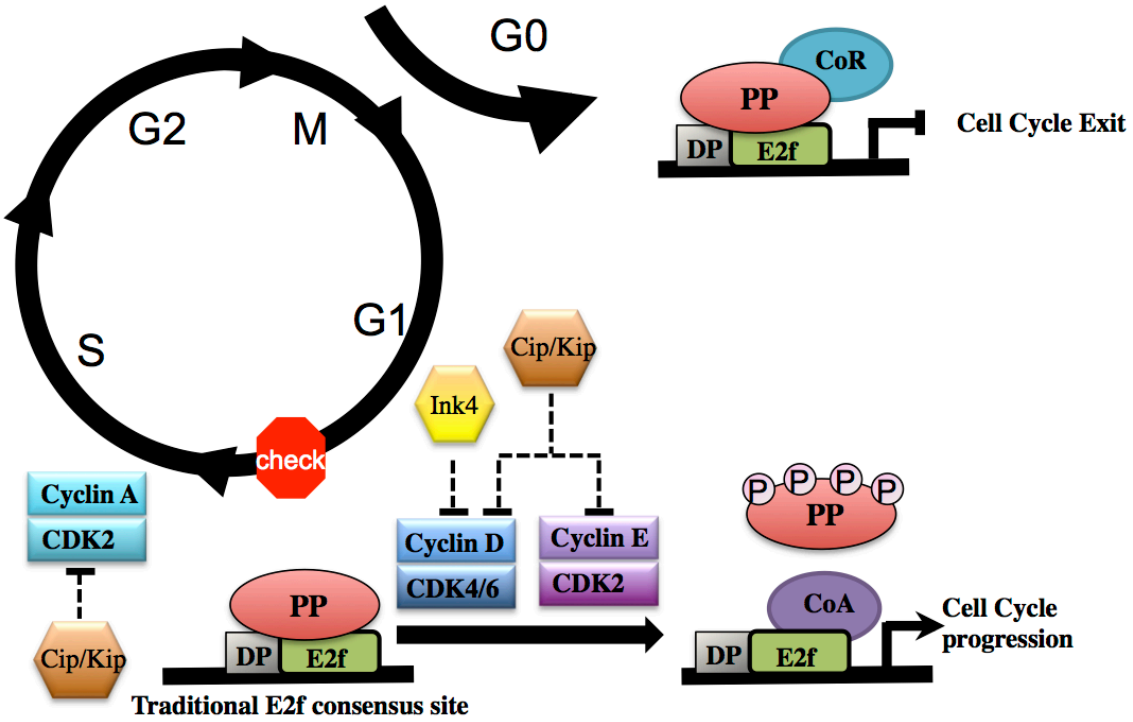


Figure 1.4: Model of Rb/E2f molecular function

At early G1 phase, E2f protein heterodimerizes with DP and forms a repressive complex with pocket proteins (PP). This complex interacts at the promoter regions of target genes. As G1 phase progresses, pocket proteins are phosphorylated by cyclin/Cdk complexes and upon hyperphosphorylation, the pocket protein is released, allowing entry and progression through to S-phase. E2f is now able to start transcription of E2f target genes and during S-phase, co-activators (CoA), such as histone acetyl-transferase, are recruited to E2f transactional complexes. At G0, cells will exit the cell cycle and become post-mitotic, pocket proteins will recruit co-repressors, such as histone deacetylases, histone methyl-transferase and DNA meythyl-transferases, to the E2f complex and repress target genes. Modified from McClelland & Slack, 2007.

likely to develop a single cancer in one eye at an older age (Knudson 1971). This study provided the basis of the "Knudson two hit hypothesis" where, in the case of familial retinoblastoma, a child would inherent a mutation, however, the cancer would only occur once a second mutation was acquired, resulting in the loss of heterozygosity. The retinoblastoma gene (*RB1*) was then characterised as a tumour suppressor due to its genetic association to inherited retinoblastoma in children (Friend, Bernards et al. 1986, Fung, Murphree et al. 1987). However, it was not until molecular cloning methods were established that the link was solidified on a mechanistic level. The retinoblastoma protein was the first tumour suppressor to be cloned (Lee, Bookstein et al. 1987). Molecular cloning of cancer-related genes such *as RB1* has advanced genetic cancer research tremendously. Later research has linked mutations in *RB1* to other cancers, such as small-cell lung carcinoma and osteosarcoma (Harbour, Lai et al. 1988, Egan, Bayley et al. 1989, Horowitz, Park et al. 1990). The overexpression of *Rb1* causes cells to arrest at the G1-phase of the cell cycle (Huang, Yee et al. 1988, Goodrich, Wang et al. 1991, Qin, Chittenden et al. 1992), while acceleration of the G1 transition is observed in *Rb1* deficient cells, supporting the hypothesis that pRb is a regulator of cell proliferation (Herrera, Sah et al. 1996, Hurford, Cobrinik et al. 1997, Classon, Salama et al. 2000).

1.4.2 Family of pocket proteins

The retinoblastoma protein is part of a family containing two other members, p107 (also known as RBL1) and p130 (also known as RBL2). All three phosphoproteins share structural homology within "their pocket domain" and are referred to as pocket protein family members. p107 and p130 are structurally closely related to one another

(~50% amino acid identity), but also to pRb (~35% amino acid identity) (Ewen, Xing et al. 1991, Hannon, Demetrick et al. 1993, Li, Graham et al. 1993). As with pRb, p107 and p130 were originally identified through their interaction with viral oncoproteins such as: adenovirus E1A (Ad-E1A) (Whyte, Buchkovich et al. 1988, Egan, Bayley et al. 1989), simian virus large T antigen (SV40 LT-antigen) (DeCaprio, Ludlow et al. 1988, Dyson, Howley et al. 1989), and the E7 human papilloma virus (HPV-E7) (Munger, Werness et al. 1989). The pocket protein family has been shown to play a pivotal role in cell proliferation. Overexpression of all three members of the pocket protein family has been shown to arrest certain cell types at the G1-phase of the cell cycle and this can be overcome by co-expression and interaction with the E2fs (Qin, Livingston et al. 1995, Vairo, Livingston et al. 1995, Moberg, Starz et al. 1996). Even though, they share similar structural homology and can bind E2fs, studies have shown all three-pocket proteins have non-redundant functions, which will be discussed in further sections.

1.4.3. E2f family of transcription factors

Historically, E2fs were identified by their sequence element that can bind the adenovirus E1A inducible E2 promoter DNA consensus sequence (Kovesdi, Reichel et al. 1986, Kovesdi, Satake et al. 1987). The first E2f to be identified was *E2f1*, which is a classical member of the E2f family, followed by seven additional members in the mammalian system (*E2f2-8*) (Figure 1.5). E2fs, associated with pocket protein family members, take part in cell cycle transcriptional activity. The large family of E2f transcription factors are best known for their role in the regulation of cell cycle and DNA synthesis genes required for proper cell cycle progression [Reviewed in (Dyson 1998,

Trimarchi and Lees 2002)]. Direct interactions between E2fs and pocket proteins can modulate gene expression at various E2f responsive sites (Burkhart and Sage 2008, Chen, Tsai et al. 2009). Rb/E2f regulates the G1-S checkpoint in basically all cell types across all species, making this a highly pervasive and relevant model to study.

We can divide the E2f family of transcription factors into two categories based on their structural features: (1) Classical E2fs (E2f1-6) contain one characteristic conserved DNA binding domain (DBD), which requires heterodimerization with one of three Differentiation-related Polypeptide proteins (DP), DP1, DP2 and DP3 to bind to its DNA (Bandara, Buck et al. 1993, Girling, Partridge et al. 1993, Trimarchi and Lees 2002, Attwooll, Lazzerini Denchi et al. 2004) and (2) atypical E2fs (E2f7-8) bear two DBD and bind DNA independent of DP protein (Figure 1.5) (Di Stefano, Jensen et al. 2003, Logan, Graham et al. 2005). Interestingly, the newly characterised atypical branch of the Rb/E2f pathway, E2f7 and E2f8, has the unique ability to bind DNA in a DP-independent (de Bruin, Maiti et al. 2003, Di Stefano, Jensen et al. 2003, Christensen, Cloos et al. 2005, Li, Ran et al. 2008, Moon and Dyson 2008). Not much is known of the tissue-specific role of atypical E2fs: the majority of what we currently know about atypical E2fs function is derived from *in vitro* and *in vivo* experiments focused on their differentiation role with the canonical Rb/E2f pathway. In section 1.5 and 1.6, I will summarize the role of atypical E2fs, defined from earlier studies, and will discuss their potential role in CNS development.

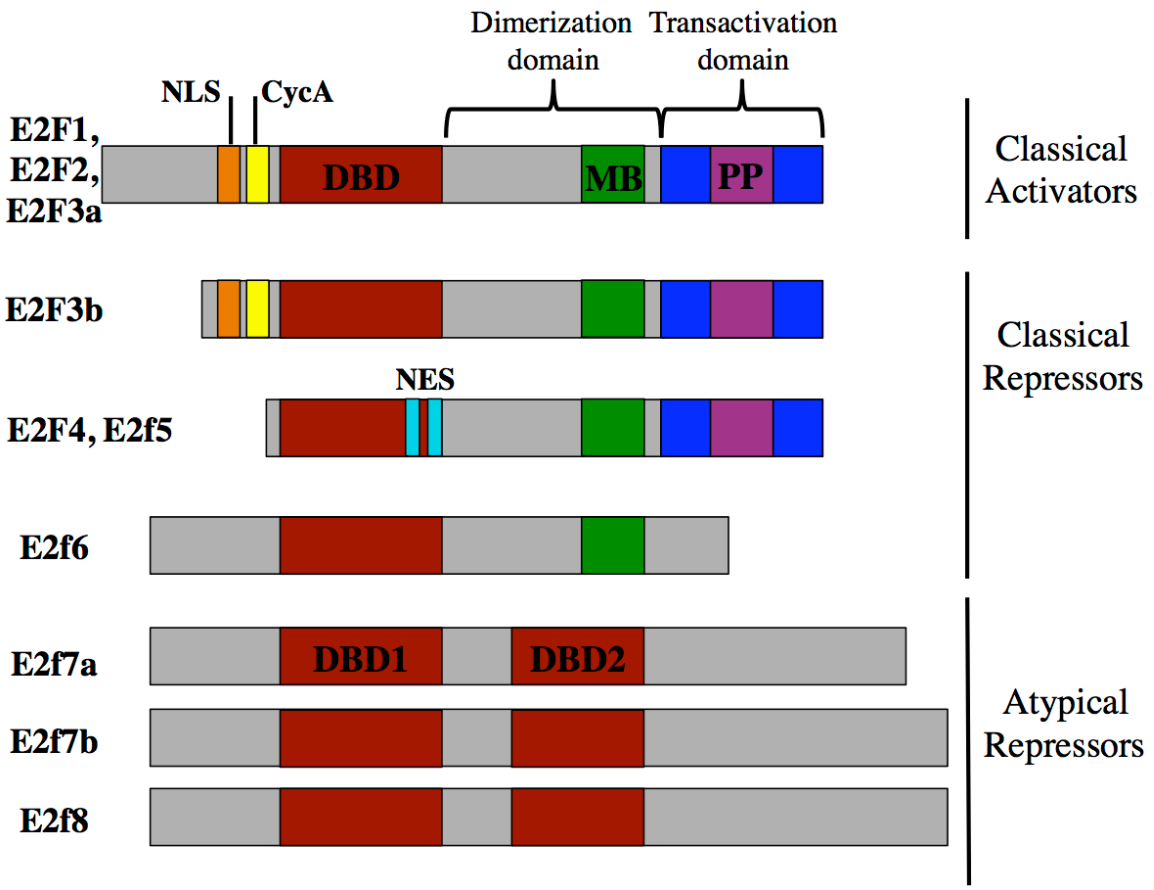


Figure 1.5: Structural diagram of E2f family members

All E2f family members contain DNA binding domain and classified as activator, classical repressors and atypical repressors. Atypical repressors, E2f7 and E2f8, contain, unlike the rest of the family members, not one but two DNA binding domains and function independent of DP. Classical E2fs, E2f1-6, contain a dimerization with DP domain which encompasses a marked box. Transactivation domain and pocket protein binding are present only in E2f1-5. E2f1-3 shares a nuclear localization sequence and a cyclin A binding domain, while E2f4-5 have a nuclear export signal instead. E2f3 encodes for two isoform, E2f3a and E2f3b, while two E2f7 proteins exist due to splicing of the E2f7 transcript. NLS= nuclear localization sequence, NES= nuclear export sequence, DBD= DNA binding domain, MB= marked box domain, PP= pocket protein binding domain. Modified from Chen et al., 2009.

1.4.4. Mechanism of action of cell cycle regulation by canonical Rb/E2f

The cell cycle is comprised of four distinct phases: the gap-1 phase (G1), the DNA synthesis phase (S-Phase), the gap-2 phase (G2) and mitosis (M-phase). DNA checkpoints throughout the cell cycle regulate completion of cell cycle events implicated in DNA replication and mitosis. For example, if DNA damage occurs during the G1-phase, signal transduction pathways mediate blockage of the S-phase events and prevents entry into mitosis (Nyberg, Michelson et al. 2002). The canonical Rb/E2f pathway is a key regulator of the G1-S transitional phase of the cell cycle (Figure 1.4).

Under non-growth factor stimulated conditions, pRb is hypophosphorylated and physically binds E2fs and represses their transcriptional activity. Progression from the G1 to S-phase occurs through sequential pRb phosphorylation events. Cyclins and cyclin-dependent kinases (CDKs) will form complexes to phosphorylate pRb (Buchkovich, Duffy et al. 1989, Cooper and Shayman 2001). Cyclin D binds CDK4/6 to initiate phosphorylation at the G1-phase, followed by cyclin E binding to CDK2 and finally the cyclin A/CDK2 complex will phosphorylate pRb at the end of the G1-phase through the beginning of the S-phase (Ezhevsky, Nagahara et al. 1997, Lundberg and Weinberg 1998). Cyclin-dependent kinase inhibitors (CKI) are negative regulators of the cyclin/CDK complexes. CKI can be subdivided into two families: 1) Ink4/Arf ($p16^{Ink4}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$), which regulate Cyclin D/CDK4/6 and, 2) Cip/Kip ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{kip2}$), which regulate all three G1-phase cyclin/CDK complexes [reviewed in (Sherr and Roberts 1999)]. When pRb reaches a hyperphosphorylated state, pRb is released from E2f-DNA complexes, allowing transcription of E2f target genes required for DNA synthesis and cell cycle progression. Based on this mechanistic canonical

model, the Rb/E2f complex is most abundant during cellular quiescence, while "free" E2fs, unbound from the pocket proteins, are abundant in proliferating cells [reviewed in (Dyson 1998)]. However, the pocket proteins are also known as transcriptional co-factors, which can repress or activate several transcription factors (Morris and Dyson 2001, Macaluso, Montanari et al. 2006). Furthermore, they can recruit chromatin-remodelling enzymes to regulate expression of target genes and modify chromatin structure (Brehm and Kouzarides 1999, Gonzalo and Blasco 2005). In human embryonic stem cells, both *RB1* deficiency and over-expression drives cell cycle arrest leading to death (Conklin, Baker et al. 2012). These findings reflect a multifaceted role of the Rb/E2fs pathway and thus, necessitate a more complex model then what the canonical model entails.

1.4.5. Comparative role of pocket protein family members

The pocket proteins contain many binding domains that can be utilized to assemble protein complexes. The pocket domain, has since then been shown to physically interact with a variety of cellular proteins. One of the best described interactions is with E2f, and this interaction is known to recruit co-repressor proteins that can modify histone marks and the chromatin structure (Luo, Postigo et al. 1998, Rubin 2013). Even though they share a high degree of sequence identity within the pocket domain, the pocket proteins are differentially expressed during the cell cycle. pRb is expressed in both quiescence and proliferating cells, while p107 is most abundant in proliferating cells and p130 is predominantly expressed in cells that have exited the cell cycle (Beijersbergen, Kerkhoven et al. 1994, Kiess, Gill et al. 1995, Garriga, Limon et al. 1998, Smith, Leone

et al. 1998, Burkhart and Sage 2008). Early i*n vivo* evidence support non-redundant functions for pocket proteins.

pRb is the member of the pocket protein family that displays the most severe phenotype. *Rb1*$^{-/-}$ mice are embryonic lethal (Clarke, Maandag et al. 1992, Jacks, Fazeli et al. 1992, Lee, Chang et al. 1992), while *p107*$^{-/-}$ and *p130*$^{-/-}$ null animals appear to develop normally (Cobrinik, Lee et al. 1996). *Rb1* homozygous mutant mice die between E13-E15 and display massive apoptosis throughout the CNS and ectopic mitosis (Jacks, Fazeli et al. 1992, Lee, Chang et al. 1992, Morgenbesser, Williams et al. 1994, Jacks 1996, Zacksenhaus, Jiang et al. 1996). It has been shown that the proliferation defect observed in *Rb1* null mutant mice is cell-autonomous, while the widespread apoptosis defect in the CNS and hematopoietic organs are related to the lack of oxygen due to a placental defect (Ferguson, Vanderluit et al. 2002, de Bruin, Wu et al. 2003, MacPherson, Sage et al. 2003, Wu, de Bruin et al. 2003). However, studies show that cell death is tissue-specific since work in lens (Liu and Zacksenhaus 2000, de Bruin, Wu et al. 2003), skeletal muscles (Zacksenhaus, Jiang et al. 1996, de Bruin, Wu et al. 2003, Huh, Parker et al. 2004) and the retina (MacPherson, Sage et al. 2003, Chen, Livne-bar et al. 2004, Chen, Opavsky et al. 2007, Chen, Chen et al. 2013) all illustrate a cell autonomous death upon *Rb1* loss. *E2f1* and *E2f3* are also associated with pRb-regulated apoptosis. In *Rb1* mutant mice, simultaneously losing *E2f1* and *E2f3* leads to a dramatic reduction in cell death (Guo, Yikang et al. 2001, Simpson, MacLaurin et al. 2001). To study the role of pRb in CNS development and processes, *Rb1* was deleted in a tissue specific manner. Deletion of *Rb1* specifically within the telencephalon, using a strategy in which Cre recombinase is expressed from the brain factor 1 (*Bf-1*) promoter, leads to ectopic

proliferation and increased neurogenesis at E16.5, without an increase in apoptosis (Ferguson, Vanderluit et al. 2002, Ferguson, McClellan et al. 2005). Furthermore, pRb directly regulates key differentiation genes in the developing telencephalon (Ghanem, Andrusiak et al. 2012). Interestingly, these brains also displayed aberrant migration defects of interneurons (Ferguson, Vanderluit et al. 2002, Ferguson, McClellan et al. 2005). Neogenin, a gene involved in neuronal migration, is directly regulated by pRb through E2f3 and is one contributor to the cellular migration defects observed in these mice (Andrusiak, McClellan et al. 2011). Compound knockout of *E2f3* is sufficient to rescue the migration defects observed in the *Rb1* telencephalon specific knockout mouse (McClellan, Ruzhynsky et al. 2007)

Unlike *Rb1* mutants, which display severe developmental defects, *p107* and *p130* null mice are viable and display comparatively mild phenotypes. Interestingly, *p107* mutants show an increase in the number of neural progenitors at the expense of neuronal differentiation in the adult SVZ (Vanderluit, Ferguson et al. 2004, Vanderluit, Wylie et al. 2007). p107 is therefore required to limit expansion of the neural progenitor pool while promoting neural cell differentiation. Mice deficient in both *p107* and *p130* die shortly after birth with severe chondrocyte and epidermal differentiation defects (Cobrinik, Lee et al. 1996, Ruiz, Santos et al. 2004). Knockout of all three pocket protein members' results in death between E9.5 and E11.5, and these embryos display massive apoptosis and a high increase in proliferation (Wirt, Adler et al. 2010). Studies show pRb plays a role in apoptosis, cell proliferation and differentiation but it is also clear that pRb, p107 and p130 can partially compensate for each other.

1.4.6. Comparative role of E2fs

Initial *in vitro* studies regarding the function of E2f family members suggest that E2fs have largely redundant roles. Cloning and functional analysis of each individual E2f factor has since shown that E2fs can have overlapping functions, yet their roles are much more complex with many factor-specific functions. Limited by sharing a similar DBD, the E2f family can greatly differ in their degree of structural homology [Reviewed in (Trimarchi and Lees 2002, McClellan and Slack 2007, van den Heuvel and Dyson 2008, Chen, Tsai et al. 2009)] (Figure 1.5). E2f1-6, in its N-terminus, contains one DBD and one dimerization domain, followed by a conserved marked box domain that facilitates dimerization with DP and DNA bending. Only E2f1-5 possesses a trans-activation domain at the C-terminus, which harbours a pocket protein binding site, permitting interactions with pRb, p107 and p130. The *E2f3* gene encodes two isoforms, E2f3a and E2f3b, which can exhibit unique expression patterns. E2f3a is the longer of the two isoforms, where E2f3b is missing a ubiquitin targeting domain at its N-terminus (Leone, Nuckolls et al. 2000). E2f1-3 contains a cyclin A binding domain and a nuclear localization signal (NLS), while E2f4 and E2f5 have a nuclear export signal (NES) and need to dimerize with DP to translocate to the nucleus. Unlike E2f1-5, E2f6 does not possess a pocket protein-binding site and functions independently of pocket protein family members. The most recently discovered E2fs, E2f7 and E2f8, are known as atypical E2fs since they function independently of both DP and pocket proteins, and they contain not one but two DBDs. This allows them to form homo- and hetero-dimers with each other when binding to their target DNA consensus sites (Lammens, Li et al. 2009).

1.4.7. E2f activity: a comparison between transcriptional activation and repression

Since the discovery that pocket proteins interact with E2fs, this pathway has been of high interest in the study of cell proliferation. The E2f family are conserved from *Caenorhabditis elegans* (*C-elegans*) to mammals (Trimarchi and Lees 2002, Attwooll, Lazzerini Denchi et al. 2004). Many genes involved in the G1/S transition have E2f-responsive binding sites and are known as E2f target genes. Such targets include: *cyclin A*, *cyclin E*, *p107*, *Dhfr*, *TK1*, *DNA pol α* and *E2f1* [reviewed in (Dyson 1998, Hallstrom and Nevins 2009)]. Modern techniques such as DNA oligonucleotide microarrays, chromatin immunoprecipitation (ChIP), RNA-Seq and ChIP-on-Chip have identified many E2f target genes beyond the G1/S transition. These 'non canonical' E2f target genes are involved in broad biological functions, including mitosis, DNA damage repair, survival, apoptosis, differentiation and development (Ishida, Huang et al. 2001, Muller, Bracken et al. 2001, Polager, Kalma et al. 2002, Stanelle, Stiewe et al. 2002, Young, Nagarajan et al. 2003, Chen, Pacal et al. 2009, Chong, Wenzel et al. 2009, Julian, Liu et al. 2015).

Like *C-elegans* and *Drosophila melanogaster*, the mammalian E2f family can be further subdivided into two classes that differentially regulate transcription: activators and repressors. Nematodes and flies contain a single E2f for each activator and repressor, while, in mammals' activators and repressors are subdivided between the eight members (Dynlacht, Brook et al. 1994, Sawado, Yamaguchi et al. 1998). The activating E2fs in mammals consist of *E2f1*, *E2f2*, *E2f3*, while the repressing E2fs include *E2f4*, *E2f5*, *E2f6*, *E2f7* and *E2f8*. In the canonical view of Rb/E2f function, the E2f4 or E2f5 co-repressors, in complex with a pocket protein, predominantly p130, occupy E2f-

responsive genes in quiescent and early G1-phase cells (Figure 1.6) (Takahashi, Rayman et al. 2000, Rayman, Takahashi et al. 2002, Ren, Cam et al. 2002). During the G1-phase, pocket proteins are phosphorylated which disassemble the repressive complex allowing E2f4 or E2f5 to be exported out of the nucleus, thus making room for activator E2fs, such as E2f1-3, to bind the promoter of genes involved in cell cycle control and DNA replication. E2f1-3 levels are elevated at G1-S phase boundary and they primarily bind S-phase target genes (Takahashi, Rayman et al. 2000, Rayman, Takahashi et al. 2002). On completion of S-phase, G1-S phase target genes are repressed, which coincides with the upregulation of E2f7-8 expression in late S-phase (Di Stefano, Jensen et al. 2003, Zhu, Giangrande et al. 2004, Christensen, Cloos et al. 2005). The sequential switch between activator and repressor E2f complexes has become a popular model.

1.4.7.1. Activator E2fs

E2f1, *E2f2* and *E2f3* are classified as activating E2fs. These E2fs have increased expression levels during cellular proliferation and during cell cycle entry, and their recruitment of histone acetyl transferases permits expression of pro S-phase genes (Trouche, Cook et al. 1996, Takahashi, Rayman et al. 2000). Overexpression of all three activating E2fs is enough to induce cell cycle entry (Johnson, Schwarz et al. 1993, DeGregori, Kowalik et al. 1995, Lukas, Petersen et al. 1996). In MEFs, deletion of *E2f1*, *E2f2* and *E2f3* leads to cell cycle arrest, loss of proliferation and reduced expression of genes responsible for G1/S transition and DNA synthesis (Wu, Timmers et al. 2001). However, in the absence of all three activating E2fs (E2f1-3), cell division continues in

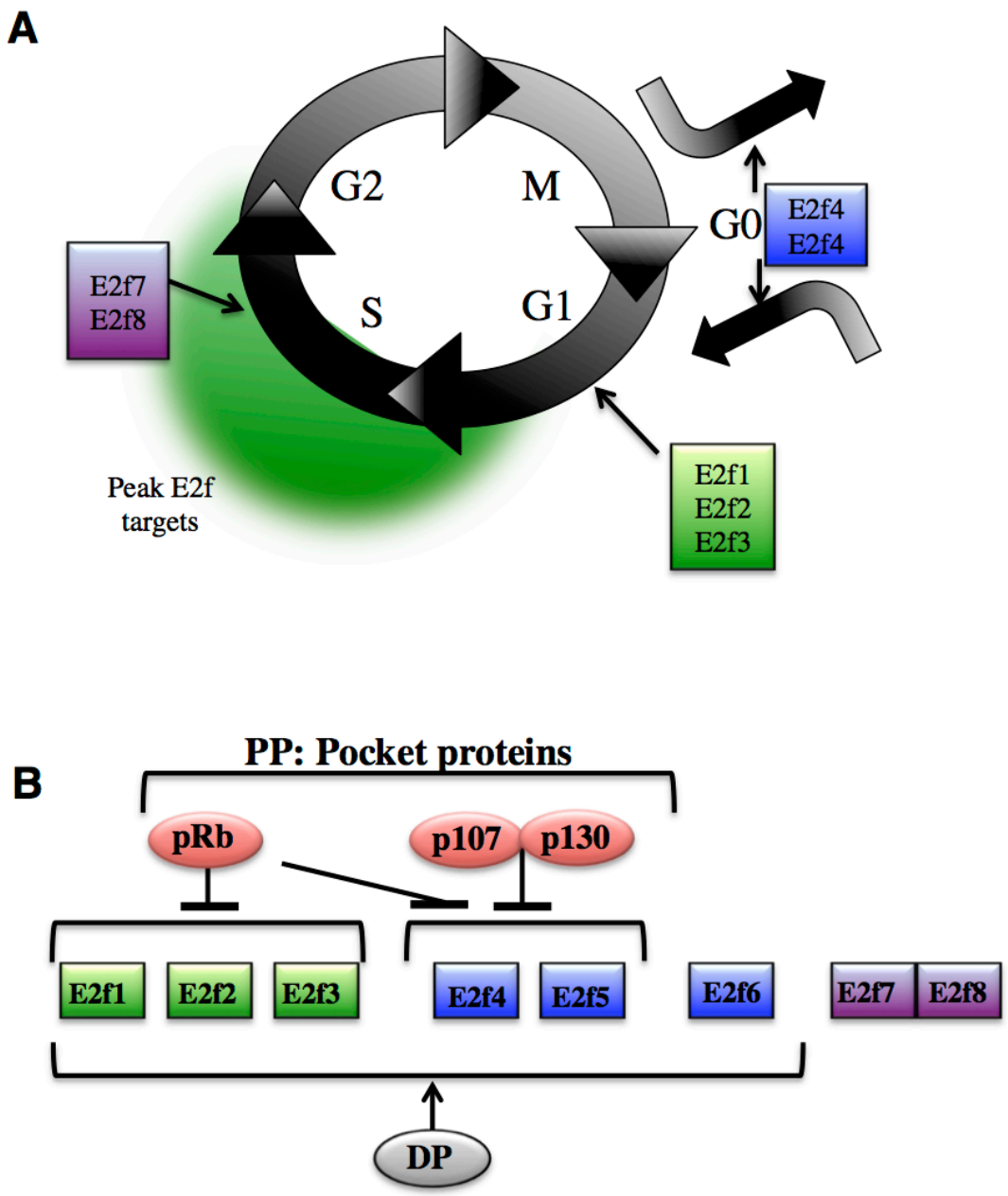


Figure 1.6: Expression and activity of Rb/E2f pathway during the cell cycle

(**A**) During G0, E2f4 and E2f5 associated with pocket proteins are ubiquitiously expressed and repress E2f target genes involved in DNA synthesis and cell cycle progression. Accumulation of activator E2fs, E2f1, E2f2 and E2f3, during G1 phase initiates transcription of E2f-mediated genes, where E2f target gene peaks during S-phase (green haze). S/G2 –transition, repressors, E2f7 and E2f8 function independently of pocket proteins and mediate repression of E2fs target genes. (**B**) Interaction between pocket proteins, pRb, p107 and p130, and the E2fs in mammals. E2f1-5 interacts with the pocket proteins, while E2f1-6 dimerize with DP. E2f7 and E2f8 function independently of pocket proteins and DP. Modified from Chen, Tsai & Leone, 2007 and van den Heuvel & Dyson, 2008.

the retina *in vivo* (Chen, Opavsky et al. 2007, Chen, Pacal et al. 2009). In the Chen et al., (2009) study, Mycn drives proliferation of retinal NPCs in E2f-independent fashion by preventing p53 induction of cyclin-dependent kinase inhibitor, *p21*. In retinal progenitors, both gain-or loss-of-function in activating E2fs leads to survival defects in retinal NPCs (Chen, Pacal et al. 2009, Chong, Wenzel et al. 2009). Both E2f3b activation and E2f3a/p107 repressor complex maintain NPCs homeostasis by transcriptional regulation of the same target gene, *Sox2* (Julian, Vandenbosch et al. 2013). In this case, the maintenance between self-renewal and differentiation is cell cycle-independent.

The classical role of activating E2fs is to promote cell proliferation. However, activating E2fs have a role beyond cell proliferation, they also have a well-known role in survival and apoptosis (Field, Tsai et al. 1996, Yamasaki, Jacks et al. 1996, Chen, Opavsky et al. 2007, Chen, Pacal et al. 2009). Mimicking a loss in *Rb1*, *E2f1* overexpression induces caspase-dependent apoptosis (Phillips and Vousden 2001, Nahle, Polakoff et al. 2002, Pediconi, Ianari et al. 2003, Iaquinta and Lees 2007). *Rb1* null mice die mid-gestation and display massive levels of apoptosis throughout the CNS. Deletion of *E2f1* or *E2f3* in the *Rb1* null background drastically reduces cell death (Yamasaki, Bronson et al. 1998, Ziebold, Reza et al. 2001). A plethora of apoptotic and DNA damaged genes have been shown to contain E2f consensus sites. These genes include *Apaf1*, *Chk1*, *PCNA* and *Fen1* (Ishida, Huang et al. 2001, Muller, Bracken et al. 2001, Polager, Kalma et al. 2002, Ren, Cam et al. 2002, Julian and Blais 2015).

Single knockout animal models have been generated for each member of the E2f family, and many of these mouse models exhibit developmental or neurogenic defects in

the brain. *E2f1*$^{-/-}$ mice display ectopic NPC proliferation as well as a decrease in both adult hippocampal and SVZ neurogenesis (Cooper-Kuhn, Vroemen et al. 2002). However, *E2f1*$^{-/-}$ mice show no abnormalities in embryonic cortical neurogenesis or in the number of embryonic and adult cortical neurons, which suggests E2f1 is dispensable for corticogenesis. On the other hand, *E2f3*$^{-/-}$ mice do display developmental defects. However the degree of severity is mouse-strain dependent (Humbert, Verona et al. 2000, Cloud, Rogers et al. 2002). These animals die at birth from congenital heart failure (Cloud, Rogers et al. 2002). Two protein isoforms are transcribed from the *E2f3* locus, and it has recently been shown that *E2f3a* and *E2f3b* control the balance between self-renewal and differentiation of NPCs through the differential regulation of *Sox2* (Julian, Vandenbosch et al. 2013). E2f3a, likely in a complex with p107, suppresses *Sox2* expression thus permitting neuronal commitment and neurogenesis, while E2f3b activates *Sox2* expression in collaboration with DNA Polymerase II to promote neural precursor expansion (Julian, Vandenbosch et al. 2013). In the retina, Rb and E2f3a regulate differentiation of starbust amacrine cells in a cell cycle independent fashion (Chen, Opavsky et al. 2007). These findings reveal a dynamic role of pocket proteins and E2fs in CNS development.

1.4.7.2. Repressor E2fs

The E2f repressor family comprises *E2f4-E2f8*. The expression levels of the repressor E2fs are elevated in quiescent and post-mitotic cells, coincident with their role in maintaining a non-proliferative state (Wu, Timmers et al. 2001, Trimarchi and Lees 2002, Chen, Pacal et al. 2009). Contrary to the activating E2fs, *E2f4* and *E2f5*

overexpression cannot overcome cell cycle arrest to induce cell proliferation (Ikeda, Jakoi et al. 1996, Mann and Jones 1996, Moberg, Starz et al. 1996). Instead, they are predominantly linked to repression of proliferation; for example, MEFs with deletion of *E2f4* and *E2f5* cannot exit the cell cycle in the presence of growth arrest signals (Bruce, Hurford et al. 2000, Gaubatz, Lindeman et al. 2000).

The typical repressor E2fs have very distinct roles compared to the activator E2fs in the context of mammalian CNS development. Although *E2f1*, *E2f2* and *E2f3* (depending on the mouse strain) single knockout mice are viable, *E2f4* single knockout animals die in early postnatal stages with cranio-facial defects and severe impairment in embryonic development, specifically of the ventral telencephalon (Humbert, Rogers et al. 2000, Rempel, Saenz-Robles et al. 2000, Ruzhynsky, McClellan et al. 2007). E2f4 controls ventral telencephalon development through genetic regulation of the Sonic Hedgehog (Shh) pathway (Ruzhynsky, McClellan et al. 2007). The Shh pathway is a morphogen linked to ventral telencephalon specification, and is a key player in proliferation and cell fate decisions of NPCs (Dessaud, McMahon et al. 2008). By E11.5, *E2f4*$^{-/-}$ embryos display a loss of ventral telencephalic structures, specifically the GE, and a corresponding decrease in expression of ventral specific homeodomain markers (Nkx2.1, Dlx1/2 and Lhx6) as well as Shh and its target *Gli1* (Ruzhynsky, McClellan et al. 2007). Knocking out one allele of *Gli3*, a Shh-dependent transcriptional repressor, can rescue the ventral telencephalon development defects in *E2f4*$^{-/-}$ embryos (Ruzhynsky, McClellan et al. 2007).

The atypical E2fs are quite unique, as they do not hold many of the features found in most of the other E2fs. Regulating transcription independent of pocket proteins, initial

studies have shown knocking out atypical E2fs in plants and mammals leads to upregulation of several E2f-regulated genes. Both E2f1 and E2f4 can occupy the *E2f7* promoter, and *in vivo* studies have shown that E2f7 binds many E2f-responsive genes and overexpression of *E2f7* downregulate the expression of many E2f target genes and can provoke G1 arrest (Blais and Dynlacht 2004, Li, Ran et al. 2008, Chen, Ouseph et al. 2012, Ouseph, Li et al. 2012), thus classifying E2fs as repressors (Ramirez-Parra, Lopez-Matas et al. 2004, Vlieghe, Boudolf et al. 2005, Li, Ran et al. 2008, Zalmas, Zhao et al. 2008).

In summary, as major regulators of the cell cycle, the canonical Rb/E2f pathway has been broadly studied in the context of cancer biology, cell cycle, apoptosis and differentiation in a wide range of organisms and mammalian tissues. In the next section, I will discuss the role of atypical E2fs. Since the identification of the atypical E2fs, initial studies have hypothesised the atypical E2fs' primary role is to suppress the activity of the canonical E2fs' activator or to compensate for the loss of *Rb1* in tissue specific context. Not much is known on the functional role of atypical E2fs in a tissue-specific context, specifically in the development of the CNS. In the following sections, I will discuss the known roles of atypical E2fs.

1.5. Atypical E2fs: Overview

The DBD sequence of E2fs is highly conserved and analysis of new proteins with similar DBD sequences permitted the first identification of three atypical E2fs in the plant species *Arabidopsis thalina*, DP-E2F-Like (*DEL1*, *DEL2* and *DEL3*) (Kosugi and Ohashi 2002, Mariconti, Pellegrini et al. 2002, Vandepoele, Raes et al. 2002). This

discovery was followed by the identification of two new E2f family members in mammals, *E2f7* and *E2f8* (de Bruin, Maiti et al. 2003, Di Stefano, Jensen et al. 2003, Logan, Delavaine et al. 2004, Christensen, Cloos et al. 2005, Logan, Graham et al. 2005, Maiti, Li et al. 2005). *E2f7* and *E2f8* orthologs were also identified in *C-Elegans* (worm), *EFL3* (Winn, Carter et al. 2011), and *Oryza sahiva* (rice) (Lammens, Li et al. 2009).

E2f7 and E2f8 share a much lower degree of homology with the other E2fs and lack specific functional domains compared to the classical E2f family members (Figure 1.5). The tandem DBDs found in these proteins permits atypical E2fs to bind DNA in a DP-independent manner; mutations in either one of the DBDs abolishes their binding to DNA (Li, Ran et al. 2008). *In vitro*, co-immunoprecipitation experiments with epitope-tagged E2f7 and E2f8 shows that they form both homo- and hetero-dimers to bind DNA (Di Stefano, Jensen et al. 2003, Maiti, Li et al. 2005, Li, Ran et al. 2008). E2f7/E2f7 homodimers are typically the most abundant species, followed by E2f7/E2f8 heterodimers, and the least preferred is E2f8/E2f8 homo-dimerization. Phenotypic characterisation of the various combinations of atypical E2f-deficient mice is in concordance with these preferred interactions. $E2f7^{+/-};E2f8^{-/-}$ knockout mice can form E2f7 homo-dimers and have no visible defects, while $E2f7^{-/-};E2f8^{+/-}$ animals, where only E2f8 homo-dimerization is possible, die within three month of postnatal life (Li, Ran et al. 2008). Upregulation of many E2f-related genes are observed in the absence of atypical E2fs, thus classifying them as transcriptional repressors.

1.5.1. Transcriptional regulation by E2f7 and E2f8

E2f7 is known to occupy the promoter of *E2f1, E2f2, E2f3, E2f4* and *E2f8*, and at least 70% of E2f7 binding sites are located in the promoter region and contain at least one classical E2f binding motif (TTTSSCGS (where S can be a G or a C)) (Westendorp, Mokry et al. 2012). Deregulation of the E2f pathway can lead to many adverse phenotypes. For example, E2f1 is needed to drive cellular proliferation, however, too much E2f1 can trigger cell death (Phillips and Vousden 2001). Expression activating E2fs (E2f1-3) peak during the G1/S transition, typical repressor E2fs (E2f4-5) stay constant, while expression of atypical E2fs peak at late S-phase into G2 transition (de Bruin, Maiti et al. 2003, Di Stefano, Jensen et al. 2003, Logan, Delavaine et al. 2004, Christensen, Cloos et al. 2005, Logan, Graham et al. 2005, Maiti, Li et al. 2005). In mammals, E2f1 is a known target of *E2f7* and *E2f8*. Deletion of both atypical E2fs can perturb E2f1 expression at the S/G2 transition (Li, Ran et al. 2008, Zalmas, Zhao et al. 2008). Double ablation of *E2f7* and *E2f8* in mice leads to widespread apoptosis, and can be rescued by removing *E2f1* apoptosis (Li, Ran et al. 2008). Atypical E2fs regulate *E2f1* expression through a cell cycle dependent feedback loop (Li, Ran et al. 2008, Moon and Dyson 2008) (Figure 1.6A).

Gain and loss of function experiments have shown that most of the genes regulated by E2f7 are in the G1/S transition and are known E2f targets. Examples of such genes include *Cdc6, Mcm2* and *Rads1* (Westendorp, Mokry et al. 2012). E2f7 expression peaks at late S-phase, which correspond to G1/S transition decline of E2f target genes. Ablation of atypical E2fs in MEFs accelerates S-phase progression (Li, Ran et al. 2008), while overexpression of E2f7 in quiescent cells entering the cell cycle will

arrest cells at S-phase (Westendorp, Mokry et al. 2012). Acute disruption of *E2f7* during the G2/M transition does not arrest cell cycle progression nor deregulate E2f target genes associated with G2/M, supporting the model that atypical E2fs control S-phase progression by regulating E2f targets specifically at the G1/S transition (Westendorp, Mokry et al. 2012). As the role of atypical E2fs in cell cycle progression has been conducted mostly in synchronised cells in culture, not much is known regarding their targets beyond cell cycle progression.

1.5.2. Known biological functions of atypical E2fs

E2f7 and *E2f8* double knockout mice die mid-gestation at E11.5, and display widespread CNS apoptosis, placental and vascular defects (Li, Ran et al. 2008). Loss of *E2f1* rescues the aberrant apoptosis, but is unable to prolong embryonic survival (Li, Ran et al. 2008). Severe placental defects are observed in these double knockout mice, and trophoblast specific double knockout, yields a similar phenotype to the $E2f7^{-/-}$:$E2f8^{-/-}$, including vascular dilation, haemorrhage and death by E11.5 (Ouseph, Li et al. 2012). Mice carrying germline deletions of atypical E2fs but with a WT placenta survive embryonic development and do not display the severe developmental phenotypes observed in the double knockout or the placental specific double knockout animals (Ouseph, Li et al. 2012). Thus, the role of E2f7 and E2f8 in trophoblast stem cells is the most likely cause of mid-gestation lethality.

Loss of atypical E2fs in mice and zebrafish leads, in both cases, to leaky vasculature and aberrant vascular sprouting. In the zebrafish, E2f7 and E2f8 form a transcriptional complex with hypoxia-induce factor (*HIF1*) to control angiogenesis by

regulating vascular endothelial growth factor (*VEGFA*) expression (Weijts, Bakker et al. 2012). VEGFA is a known regulator of embryonic angiogenesis (Dunwoodie 2009, Bakker, Weijts et al. 2013). Although atypical E2fs are broadly classified as transcriptional repressors, in this model atypical E2fs activate *VEGFA* expression through a HIF1 binding site in the *VEGFA* promoter (Weijts, Bakker et al. 2012). Double knockout *E2f7* and *E2f8* mice have a similar phenotype to the *HIF1* knockout mice, whereby both animal models die mid-gestation (Dunwoodie 2009).

E2f7 and E2f8 share common expression patterns among embryonic and adult tissues. Both have high expression in the foetus and placenta during embryonic development. In adult mice, high expression can be seen in the skin, thymus, and spleen, while moderate expression is found in the intestines and testis, and low expression is detected in the brain, muscle, and stomach (de Bruin, Maiti et al. 2003, Maiti, Li et al. 2005).

1.5.3. The relation of atypical E2Fs with the canonical Rb/E2f pathway

As discussed above, the canonical Rb/E2f pathway is a well-known key regulator of the G1/S transition of the cell cycle, and pRb, p107 and p130, by binding to the E2fs, represses transcription. The pocket proteins cannot repress atypical E2fs directly, since atypical E2fs lack the pocket protein-binding site. An intriguing observation is that the double knockout for *E2f7* and *E2f8* has a similar phenotype to $Rb1^{-/-}$ animals (Li, Ran et al. 2008). Both animal models have widespread apoptosis that can be rescued by deletion of *E2f1*, as well as vascular and placental defects. *Rb1* knockouts also die mid-gestation by placental dysfunction and placenta-specific knockout of *Rb1* leads to excessive

trophoblast proliferation and defects in blood vessels (Wu, de Bruin et al. 2003). Based on the similarities between the two mouse models, it is likely the canonical Rb/E2f pathway and atypical E2fs interact at some level.

In human cells, oncogenic mutations induce *E2F7* via p53, and when *RB1* is also removed, *E2F7* is further induced (Aksoy, Chicas et al. 2012). It is believed E2F7 can compensate for RB to repress mitotic genes and prevent uncontrolled proliferation. During senescence or DNA damage, p21 inhibits the CDKs, thus preventing hyperphosphorylation of RB, and promoting repression of S-phase E2F target genes (Aksoy, Chicas et al. 2012). The role of E2F7 in this system is only apparent in the absence of *RB1*. In a senescent state, E2F7 is therefore capable of compensating for pRB, possibly by acting downstream to directly repress S-phase E2F target genes.

E2f8 and pRb also function synergistically to regulate erythropoiesis (Hu, Ghazaryan et al. 2012, Ghazaryan, Sy et al. 2014). Specific deletion of *Rb1* in hematopoietic stem cells (HSCs) leads to mild anemia, while both *E2f7* and *E2f8* single knockouts in HSCs show no phenotype. Double knockout of $Rb1^{-/-}$:$E2f8^{-/-}$ but not $Rb1^{-/-}$:$E2f7^{-/-}$, in this system leads to severe anemia and hemolysis (Ghazaryan, Sy et al. 2014). Inactivation of *E2f2* rescues the erythropoitic defects observed in the $Rb1^{-/-}$:$E2f8^{-/-}$ double knockout animals (Ghazaryan, Sy et al. 2014).

Atypical E2fs have an overlapping set of targets with E2f3a, and atypical E2fs and E2f3a differentially regulate these targets in extra-embryonic tissue (Chen, Ouseph et al. 2012, Ouseph, Li et al. 2012). Compound knockout of *E2f3a* can rescue the placenta defects observed in $E2f7^{-/-}$:$E2f8^{-/-}$ mice, and these animals progress through embryonic development. Like E2f1, E2f3a expression peaks at the G1/S transition and is associated

with activation of S-phase target genes, but E2f3a can also activate atypical E2fs (Li, Ran et al. 2008, Ouseph, Li et al. 2012). E2f7 and E2f8 expression increases by mid-S-phase, when E2f3a is targeted for degradation, thus repressing S-phase target genes. Together, E2f7, E2f8 and E2f3a temporally control placental development.

In the embryonic mouse telencephalon, expression of E2f7 but not E2f8 is increased in the absence of *Rb1* (Ghanem, Andrusiak et al. 2012). The deregulation of *E2f7* interferes with the machinery of neural differentiation (will be discussed in further sections). Thus, while E2f7 and E2f8 share high homology and key functional domains with one another, they also exhibit unique tissue-specific roles.

1.5.4. Atypical E2fs in CNS development

Not much is known about the function of atypical E2fs in the brain. *E2f7* and *E2f8* single knockout mice show very mild developmental phenotypes, however the double knockout animals die mid-gestation, thus suggesting atypical E2Fs are essential for development. This also confirms compensatory functions between these two E2fs (Li, Ran et al. 2008). Widespread apoptosis is observed across the CNS in the $E2f7^{-/-}$:$E2f8^{-/-}$ mice; however, simultaneous deletion of *E2f1* rescues the apoptosis. Interestingly, however, this does not prolong viability of the animals (Li, Ran et al. 2008). The lack of viability of double knockout animals has been linked to defects in placental development, where E2f7 and E2f8 are required for proper trophoblast cell proliferation (Ouseph, Li et al. 2012). Deregulation of the Rb/E2f pathway leads to ectopic expression of atypical E2fs. In the context of the brain, even though expression of atypical E2fs in the brain is normally low, loss of *Rb1* in the telencephalon leads to specific upregulation of E2f7. *In*

vitro and *in vivo* experiments have shown that E2f7 can repress the key differentiation genes *Dlx1* and *Dlx2* in the absence of *Rb1* (Ghanem, Andrusiak et al. 2012). This study is the first piece of evidence identifying a potential role of E2f7 in CNS development.

In summary, CNS development is achieved by a tightly coordinated series of events, including cellular proliferation, differentiation, survival and migration. The Rb/E2f pathway is an integral part of a cell's ability to enter the DNA synthesis phase, or to abort cell division. Initially identified as a key regulatory pathway of the G1/S cell cycle checkpoint, it is now known that the Rb/E2f pathway has many roles beyond cell cycle regulation. Rb/E2f represents a core pathway in cell cycle regulation, and our understanding of atypical E2fs in other systems will provide important insight on their unique function in CNS development.

2. RATIONALE

The retinoblastoma protein (pRb) is best known for its role in cell cycle regulation. Through direct repression of E2f transcription factors and derepression thereof, pRb controls transition through the G1/S phase of the cell cycle (MacPherson, Sage et al. 2003, McClellan and Slack 2006, Chen, Tsai et al. 2009, Freedman, Chang et al. 2009). Manipulation of the Rb/E2f pathway can directly impact both embryonic and adult neurogenesis (Ferguson, Vanderluit et al. 2002, MacPherson, Sage et al. 2003, Chen, Livne-bar et al. 2004, Ferguson, McClellan et al. 2005, Chen, Opavsky et al. 2007, McClellan, Ruzhynsky et al. 2007, McClellan and Slack 2007, Chen, Pacal et al. 2009, Chong, Wenzel et al. 2009, Andrusiak, Vandenbosch et al. 2012, Ghanem, Andrusiak et al. 2012, Chen, Chen et al. 2013, Oshikawa, Okada et al. 2013). Additionally, deregulation of the Rb/E2f pathway leads to upregulation of E2f7 and E2f8 in human oncogenic cells, in hematopoietic stem cells, and in the telencephalon (Christensen, Cloos et al. 2005, Lammens, Li et al. 2009, Ghanem, Andrusiak et al. 2012). pRb also plays a role in neuronal differentiation through the regulation of *Dlx1* and *Dlx2*. In the absence of *Rb1*, E2f7 negatively regulates *Dlx1* and *Dlx2* gene expression *in vitro* and *in vivo* (Ghanem, Andrusiak et al. 2012). Initial studies have linked atypical E2fs to processes such as apoptosis and proliferation, and their activity is regulated in a cell-cycle dependent manner (DeGregori and Johnson 2006, Moon and Dyson 2008). From our knowledge on the role of the Rb/E2f pathway in the telencephalon, a potential role for atypical E2fs in neuronal processes such as proliferation, differentiation, survival and cell death is very intriguing. The function of atypical E2fs in the brain is completely

unknown; it will be of considerable interest to determine whether atypical E2f have a functional role in CNS development.

3. HYPOTHESIS

Stemming from our knowledge on the importance of the Rb/E2f pathway in CNS development, we hypothesize that atypical E2fs plays a crucial role in telencephalic development and may influence CNS developmental processes by regulating expression of canonical Rb/E2f target genes.

4. AIMS

In order to test our hypothesis: (**1**) we will examine the endogenous role of atypical E2fs in brain development through the use of an *in vivo* mouse model in which *E2f7* and *E2f8* are deleted in neural precursor cells of the telencephalon at approximately E8.0. Based on our findings, (**2**) we will then identify the role of E2f7 and E2f8 in the regulation of SVZ neural stem cell niche. Finally, (**3**) we will determine molecular mechanisms by which atypical E2fs regulate the postnatal SVZ neural stem cell niche.

5. MATERIALS AND METHODS

5.1. Mice, colonies and BrdU injections

E2f7 floxed and *E2f8* floxed mice were generated by Gustavo Leone and Alain de Bruin (Li, Ran et al. 2008), and *Nestin-Cre* mice, were generated by pronuclear injection of the *Nestin-cre* plasmid into FVBN mouse embryos by Ruth Slack and David Picketts (Berube, Mangelsdorf et al. 2005). These were maintained on a pure FVBN background. The animals used in the experiments were generated as follows: for initial adult characterization, females or males carrying double homozygous floxed *E2f7* and *E2f8* alleles ($E2f7^{flox/flox};E2f8^{flox/flox}$) were crossed with females or males carrying triple heterozygous for *Nestin- Cre, E2f7* and *E2f8* alleles ($Nestin\text{-}Cre/^{+};E2f7^{flox/+};E2f8^{flox/+}$). This allowed the generation of double mutant (cDKO), *E2f7* mutant (cE2f7), *E2f8* mutant (cE2f8), Cre control and no-cre control (Table 5.1). All subsequent experiments, females or males carrying double homozygous floxed *E2f7* and *E2f8* alleles ($E2f7^{flox/flox};E2f8^{flox/flox}$) were crossed with females or males carrying double homozygous floxed *E2f7* and *E2f8* alleles and heterozygote for *Nestin-Cre* ($Nestin\text{-}Cre/^{+};E2f7^{flox/flox};E2f8^{flox/flox}$). This allowed the generation of double mutant cDKO and $E2f7^{flox/flox};E2f8^{flox/flox}$ (control) in the same litter.

$E2f1^{+/-}$ (M. Greenberg) and *E2f3* floxed (Leone, Sears et al. 2001) were maintained on a mixed (C57BL/6xFVBNx129sv) background. The animals used in experiments were generated as followed: females or males carrying double homozygous floxed *E2f7* and *E2f8* alleles and double heterozygous for *E2f1* and floxed *E2f3* ($E2f7^{flox/flox};E2f8^{flox/flox};E2f1^{+/-};E2f3^{flox/+}$) were crossed with females or males carrying double homozygous floxed *E2f7* and *E2f8* alleles and triple heterozygote for *E2f1*, floxed

Name	Genotype		
	E2f7-flox	E2f8-flox	Nestin-Cre
No Cre-control	Flox/+ or Flox/Flox	Flox/+ or Flox/Flox	-
Cre-control	Flox/+	Flox/+	+
cE2f7	Flox/flox	Flox/+	+
cE2f8	Flox/+	Flox/flox	+
cDKO	Flox/flox	Flox/flox	+

	E2f7-flox	E2f8-flox	E2f1	E2f3-flox	Nestin-Cre
Control	Flox/flox	Flox/flox	+/- or +/+	Flox/+	-
cDKO	Flox/flox	Flox/flox	+/-	Flox/+	+
7:8:1:TKO	Flox/flox	Flox/flox	-/-	Flox/+	+
7:8:3:TKO	Flox/flox	Flox/flox	+/-	Flox/flox	+

Table 5.1: Genotype of transgenic mice

Genotype and name for transgenic mice used in this thesis.

E2f3 and *Nestin-Cre* (*Nestin-Cre*$^{/+}$;*E2f7*$^{flox/flox}$;*E2f8*$^{flox/flox}$;*E2f1*$^{+/-}$;*E2f3*$^{flox/+}$). This allowed the generation of triple mutants 7:8:1:cTKO, 7:8:3:cTKO , cDKO and controls (Table 5.1).

The *E2f4* mice were generated by Dr. Jacqueline Lees (Humbert, Rogers et al. 2000) and were maintained on a pure C57BL/6 background. The animals used in experiments were generated as followed: females and males caring double heterozygous allele for *E2f4* were crossed together. This allowed the generation of *E2f4*$^{-/-}$, *E2f4*$^{+/-}$ and *E2f4*$^{+/+}$ littermates.

The time of plug identification was considered to be day 0.5 for all embryonic time points (E14.5 and E18.5). For commitment (E13.5-E14.5) and birthdating experiments (E13.5-18.5), pregnant mice were injected with a single injection of BrdU at 20μg/g of body weight. For the 2-hour BrdU pulse experiments, adult mice received intraperitoneal injection of BrdU at 100μg/g of body weight (Vanderluit, Ferguson et al. 2004).

All experiments were approved by the University of Ottawa's Animal Care and Ethics committee and adhere to the guidelines put forth by the Canadian Council on Animal Care. All mice were genotyped using the Sigma Extract-N-Amp kit (Sigma) and primers designed around the *E2f7, E2f8, E2f1, E2f3, E2f4* and *Cre* locus (Table 5.2) according to the manufacturer's protocol.

Genotyping Primers		
Genes	**Name**	**Sequence**
E2f7	5080AF	5'-AGGCAGCACACTTGACACG-3'
	8301DR	5'-CCAAGATGAAGGCCGAGATGCTAC-3'
	5411ER	5'-ACTTTTGGGACAGAGGTAGGA-3'
E2f8	loxPF	5'-TAAAAAGCTTTGCGGTCGTT-3'
	allele E	5'-CTCGCATCATCGTCTGCTAA-3'
	RV loxPR	5'-AAGCCAACCTCGATGAATTG-3'
Cre	Cre-3B	5'- TGACCAGAGTCATCCTTAGCG – 3'
	Cre-5B	5'- AATGCTTCTGTCCGTTTGCC – 3'
E2f1	E2f1-3	5'-CTAAATCTGACCACCAAACGC-3'
	E2f1-PGKB	5'-CAAGTGCCAGCGGGGCTGCTAAAG-3'
	E2f1-5	5'-GGATATGATTCTTGGACTTCTTGG -3'
E2f3	E2f3-A	5-' TGAATCATGGACAGAGCCAGG-3'
	E2f3-B	5'-GTGGCTGGAAGGGTGCCAAG-3'
	E2f3-C	5'-GATTGATTCTGGGTTGTCAGG-3'
E2f4	WT	5'-ACTGATTTCTAGTCCTGCTCC-3'
	WT/KO	5'-AACCCTGAACTTATCAAGCCTC-3'
	KO	5'-CTTCAATTTGTCACGTCCTGC-3'
qPCR Primers		
Genes	**Forward**	**Reverse**
E2f7	5'-GCCAAGCAGGAAACAGAAGA-3'	5'-ACCGTGCCAACCATACTGAT-3'
E2f8	5'-GAGAAATCCCAGCCGAGTC-3'	5'-CATAAATCCGCCGACGTT-3'
CDC6	5'-AGTTCTGTGCCCGCAAAGTG-3'	5'-AGCAGCAAAGAGCAAACCAGG-3'
Gli3	5'-AGATCCTAAGCCGACAGCAA-3'	5'-GTGCTGCTCACTGCAGACTC-3'
SHH	5'-GGAAAGAGGCGGCACCCCAAAAAG-3'	5'-CTCATCCCAGCCCTCGGTCACTCG-3'
GAPDH	5'-GGTGAAGGTCCGTGTGAACG-3'	5'-CTCGCTCCTGGAAGATGGTG-3'
Cloning Primers		
Gli3	5'-TGGAATTCAAGTAACAGGTTGGGGTGA GTACCT-3'	5'-CTGAATTCCTCTCCCACCGCGGAG-3'
ChIP Primers		
Gli3	5'-GGCCTGGATGTGTCTGTGT -3'	5'-TGCTCGCCTTACTTTGCTTT -3'

Table 5.2: Primers used for genotyping, cloning, and ChIP PCR

Forward and reverse primers used for genotyping purpose to amplify regions unique to E2f7, E2f8, Cre, E2f1, E2f3 and E2f4 loci, for cloning of the Gli3 promoter region for the luciferase and for amplifying the Gi3 loci from ChIP product by PCR.

5.2. Tissue Preparation and Immunohistochemistry

For embryonic time points, pregnant female mice were euthanized by injection of 19.5mg of Euthanyl (Supplied by: Animal and Veterinary Care Services, The University of Ottawa) followed by cervical dislocation. E14.5 and E18.5 embryos were dissected, euthanized by decapitation and heads were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in 1x phosphate buffered saline (PBS), pH of 7.4. Postnatal mice were anesthetized with 6.5mg of Euthanyl (P3 and P8) and adult mice with 19.5mg of Euthanyl: both by intraperitoneal injection. Brains were dissected following transcardiac perfusion with saline (0.9% NaCl in water solution) and 4% PFA and postfixed with 4% PFA at 4°C overnight. Tissue was treated and sectioned as previously described (McClellan, Ruzhynsky et al. 2007). Before antibody incubation, when necessary, antigen was performed by heating sections at 95°C for 20 minutes in Antigen Retrieval solution (DAKO), followed by 20 minutes at room temperature. For BrdU staining, the slides were further treated with 2N HCl at 37°C for 30 minutes, then neutralized with 0.1M borate buffer for 15 minutes at room temperature. Slides were then incubated overnight at 4°C in primary antibody diluted in 0.1%Triton/0.1% Tween/PBS (Table 5.3). The appropriate conjugated secondary antibody was applied and diluted in 0.1%Triton/0.1% Tween/PBS (Table 5.3). Slides were incubated at room temperature for 2 hour and then treated for 5 minutes with 1 mg/ml 4',6-diamidio-2-phenylindole (DAPI) (Sigma), diluted 1:1000 in PBS. Slides were coverslip with Immunomount (Genetex) and imaged in a Zeiss confocal microscope using Zen imaging software or fluorescent microscope with AxioVision software. Cresyl violet staining was performed as per standard protocols Sirkin (1983) and as previously described

Immunostaining		
Antibody	**Dilution**	**Source**
	Primary	
S100 (Rabbit)	1:500	DAKO
GFAP (Mouse)	1:1000	Chemicon International inc.
Nestin (Goat)	1:1000	R&D Systems
BrdU (Rat)	1:500	Accurate Chemicals
Ki67 (Mouse)	1:100	BD Pharmigen
Acetylated Tubulin (mouse)	1:1000	Sigma
AC3 (Rabbit)	1:250	Cell Signalling
Vimentin (Goat)	1:500	Santa Cruz
FoxJ1 (Mouse)	1:500	eBioscience
Sox2 (Goat)	1:500	Santa Cruz
Tbr2 (Rabbit)	1:500	Abcam
Tuj (Rabbit)	1:1500	Covance
PH3 (Rabbit)	1:500	Millipore
GFP (Chicken)	1:1000	Abcam
N-Cadherin	1:100	Abcam
E-Cadherin	1:100	Abcam
	Secondary	
Alexa Fluor-488 (donkey)	1:500	Jackson Immuno
Cy3 (donkey)	1:500	Jackson Immuno
Dylight-649 (donkey)	1:500	Jackson Immuno

Table 5.3: Antibodies used for Immunostaining

Antibodies used for this thesis immunostaining are listed, as well as their dilution and the source of the antibody is shown. Secondary antibodies were used depending on the appropriate primary conjugate. The fluorophores used, the dilution and their sources are also listed. The species in which the antibody was generated is listed in brackets.

(Ruzhynsky, McClellan et al. 2007). Slides were then coverslipped, using a Permount (Fisher) and analyzed on an Axioplan microscope with AxioVision software using an AxioVision camera (Zeiss).

5.2.1. Cell Counts and Statistical analysis

For manual cell quantification, counts were performed using confocal images on five adjacent sections from each brain that had been levelled based on their rostral caudal location using Cresyl violet staining. Values are expressed as per section, population or area. An unpaired two-tailed Student's T-test was performed for all graphs in every figure with the exception of the BrdU birthdating experiment. For the birthdating experiment a two-way ANOVA was performed on the means of results followed by a Bonferroni post hoc test. Both tests have a minimum 95% confidence threshold to detect a difference in the mean at a *P* value of <0.05.

5.2.2. Brain measurements and Statistical analysis

For brain measurements, all sections from each brain were levelled rostra-caudally based of Cresyl violet staining. Circumference of ventricles, brain and SOC was measured manually using ImageJ (Wayne Rasband, US National Institutes of Health, Bethesda). A one-way ANOVA was performed on the means of results for the percentage of the lateral ventricle (LV)/brain ratio for each animal group followed by a Tukey's post-hoc test to detect a difference in the mean at a *P* value of <0.05. An unpaired two-tailed Student's T-test was performed for the SOC experiment with a minimum 95% confidence threshold to detect a difference in the mean at a *P* value of <0.05.

5.3. *In Situ* Hybridization

In Situ hybridization (ISH) was adapted from published protocol (Wang, Dakubo et al. 2005). Tissue preparation and fixation of P3 cDKO and control animals were performed as described above. Non-radioactive ISH was conducted using Digoxigenin (DIG) labelled antisense RNA riboprobes by *in vitro* transfection from linearized plasmids pBluescript-IIKS template containing a 0.8 kb Gli3 cDNA insert (Hui and Joyner 1993, Furimsky and Wallace 2006). Briefly, sections were hybridized overnight at 65°C, washed and incubated with an alkaline phosphatase-conjugated anti-DIG antibody at a dilution of 1:1500 (Roche). Staining was performed using Nitro blue tetrazolium (Roche) and 5-bromo-4-chloro-3-indoylphsphate (Roche) and analyzed on an Axioplan microscope with AxioVision software using AxioVision camera (Zeiss). All results shown are representative of three independent animals.

5.4. Wholemount dissection, Fixation and Immunohistochemistry

Whole-mount protocol was adapted from Arturo Alvarez-Buylla (Mirzadeh, Doetsch et al. 2010). Mice were sacrificed using mechanical cervical dislocation for animals younger then P15 and euthanized using 19.5mg of Euthanyl for animals older then P15, followed by cervical dislocation. Heads were cut off, separated into two hemispheres and dissection of the SVZ was done as per protocol (Mirzadeh, Doetsch et al. 2010). SVZ from the dissection was fixed in 4% PFA at 4°C overnight. The wholemount was incubated for 1 hour in 10% normal donkey serum/0.5% triton/PBS blocking solution. Primary antibody was diluted in blocking solution and incubated at 4°C for 24-48 hours (Table 5.3). Appropriate conjugated secondary antibody was diluted

in blocking solution and incubated at 4°C for the same amount of time as primary antibody and then treated for 30 minutes with 1 mg/ml of 4',6-diamidino-2-phenylindole (DAPI) (Sigma), diluted 1:1000 in PBS. The isolated SVZ was further dissected to isolate the ventricular wall as per protocol (Mirzadeh, Doetsch et al. 2010). Wholemount were coverslipped with Immunomount (Genetex), and slides are then stored at 4°C for 1-2 days to permit them to settle and then imaged in a Zeiss confocal microscope using Zen imaging software.

5.4.1. Cell Counts and Statistical analysis

For manual cell quantification, counts were performed using z-stack confocal images for each SVZ section. Values are expressed as per population. Two-way ANOVA was performed on the means of results followed by a Bonferroni post-hoc test for all graphs in every figure with the exception of the %GFAP$^+$nestin$^+$ cells graph (Figure 6.11), which an unpaired two-tailed Student's T-test was performed. Both tests have a minimum 95% confidence threshold to detect a difference in the mean at a *P* value of <0.05.

5.5. Stereotaxic surgery and Free Floating Immunohistochemistry

Stereotaxic injections was adapted from published protocol (Tashiro, Zhao et al. 2006). All surgical procedures were performed according to the University of Ottawa's Animal Care and Ethics committee and adhered to the guidelines put forth by the Canadian Council on Animal Care. Briefly, 9 week old, adult littermates for *E2f7*$^{flox/flox}$;*E2f8*$^{flox/flox}$ or *E2f7*$^{flox/+}$;*E2f8*$^{flox/+}$, were kept under isoflurane inhalation

anesthesia for the complete duration of the surgery. Mice were sterotaxically injected with 1.5µl of a 1:1 mixture of CAG-Cre-GFP and CAG-RFP-expressing virus into the dentate gyrus and the SVZ in both the unilateral and bilateral hemispheres. Dr. Renaud Vandenbosch conducted all surgical procedures.

The animals were sacrificed 28 days post injection (dpi) and brains were collected and fixed as described above. Brains were then separated between the right and left hemisphere and frozen as preciously described. The right hemisphere was sectioned coronally at 30µm and collected as free-floating sections in PBS and stored at 4°C for future dentate gyrus analysis. The left hemisphere was sectioned sagittally at 20µm, onto Superfrost Plus slides and stored at -80°C for olfactory bulb analysis. Immunohistochemistry was described as above, using an anti-GFP antibody without the use of DAKO Antigen Retrieval solution.

Analysis of the dentate gyrus was performed through the whole extent of the rostro-caudal axis and the olfactory bulbs through the whole extent of the medial-lateral axis, using a 1-in-6 series. To analyze the ratio of co-labeled cells, we counted all virus-labeled cells within the 1-in-6 series that were GFP/RFP-expressing cells normalized to the total number of RFP-expressing cells (Table 5.3). An unpaired two-tailed Student's T-test was performed with a minimum 95% confidence threshold to detect a difference in the mean at a P value of <0.05.

5.6. Cell culture

All cells were maintained in a humidified incubator at 37°C with 5% CO_2.

5.6.1. Neurosphere assay

Neurosphere assay was adapted from published protocol (Reynolds and Weiss 1992). Dissociated cDKO and control cells to single cell suspension from murine E14.5 ganglionic eminence were plated at a clonal density of 5000 cells/well and 6 well per embryo in 500µl of stem cell medium (Table 5.4). Primary neurospheres were counted after 7 days *in vitro*. An unpaired, two-tailed Student's T-test was performed with a minimum 95% confidence threshold to detect a difference in the mean at a *P* value of <0.05.

5.6.2. Differentiation assay

Neurospheres were induced to differentiate as adapted from a published protocol (Chojnacki and Weiss 2008). Neurospheres from cDKO control and WT CD1 (Charles River) embryos at E14.5 were cultured as described above for 5 days. They were then trypsinized for 5 minutes at 37°C with TrypLE and then triturated. Cells were counted and plated on 10 cm dish at 4.5×10^6 cells per dish or in 24 well plate at 50,000 cells per well coated with poly-L-ornithine and 10µg/mL laminin. Cells were plated in a monolayer medium (Table 5.4) and were allowed to grow as a proliferative monolayer for 3 days. At this time, day 0 is collected, while the cells were changed for differentiation medium (Table 5.4). Cells were allowed to differentiate and harvested 1, 3, 5, 7 and 10 days later. A 50% medium change was performed every second day after which cells were either fixed for immunocytochemistry or scraped into a Falcon tube and centrifuged to collect the cell pellet, washed once in PBS, frozen in dry ice and stored at -80°C. A two-way ANOVA test with Bonferronie was performed with a minimum 95%

Stem Cell Medium		
Component	**Final Concentration**	**Source**
DMEM:F12	N/A	Gibco
B27	2%	Invitrogen
ABAM	1%	Sigma
Heparin	2µg/mL	Sigma
FGF	0.02µg/mL	Sigma
Monolayer Medium		
DMEM:F12	N/A	Gibco
N2	1%	Invitrogen
ABAM	1%	Sigma
Heparin	2µg/mL	Sigma
FGF	0.02µg/mL	Sigma
Differentiation Medium		
DMEM:F12	N/A	Gibco
N2	1%	Invitrogen
ABAM	1%	Sigma
Heparin	2µg/mL	Sigma
FBS	1%	Gibco
Ependymal Cell Medium		
DMEM Glutamax-I	N/A	Gibco
FBS	10%	Gibco
Penicillin Streptomycin	1%	Gibco
Ependymal Cell Differentiation Medium		
DMEM Glutamax-I	N/A	Gibco
Penicillin Streptomycin	1%	Gibco
Enzymatic Digestion Solution		
Ependymal cell medium	N/A	
Papain	3% (stock 100mg)	Worthington
DNAse I	1%	Worthington
Cystein	2.4% (stock 12mg/ml)	Sigma
Stop Solution		
Liebovitz's L-15 medium	N/A	Gibco
Trypsin Inhibitor	11% (stock 1mg/ml)	Worthington
DNAse I	1%	Worthington

Table 5.4: Cell Culture Medium

The components of the mediums used for cell culture are listed along with their working concentration and source.

confidence threshold to compare the means of each animal group and the significance was assessed at a value of $p<0.05$.

5.6.3. Ependymal cell culture

Ependymal cell culture was adapted from published protocol (Delgehyr, Meunier et al. 2015). cDKO, control and wild-type (WT) animals from P0-P3 were sacrificed using scissors for decapitation. The brains were dissected and SVZ was collected as protocol (Delgehyr, Meunier et al. 2015). SVZ was rapidly dissociated with a razor blade and added to enzymatic digestion solution and incubated 45 minutes at 37°C. Cells were then centrifuged to remove supernatant and added a stop solution. Supernatant was removed and samples were washed with Liebovitz's L-15 medium (Gibco) and mechanically dissociated. Then centrifuged once more to remove supernatant, cells were resuspended in ependymal cell medium and plated into a 25-cm^2 flasks coated with Poly-$_L$-Lysine (Sigma) (Table 5.5), followed by a complete medium change 24 hours later. When cells reach confluency, around 4-5 days, the flask is then shaken overnight at room temperature. Medium is removed and cells are washed with Ca^{2+}/Mg^{2+}-free PBS, trypsinized for 5 minutes at 37°C with trypsin-EDTA (Gibco) and then neutralized with decomplemented FBS (Gibco). Cells are resuspended in ependymal cell medium (Table 5.4) and plated at a concentration of 2 x 10^5 cell per 20µl on Poly-$_L$-Lysine coated coverslips. Samples are incubated for 1h at 37°C before adding 500µl of ependymal cell medium. The following day (Day 0), cells are rinsed and incubated in ependymal cell differentiation medium and permitted to differentiate into multiciliated ependymal cells (Table 5.4). The cells were allowed to differentiate and harvested 0, 5, and 10 days later.

A 50% medium change was performed every second day after which cells were either fixed for immunocytochemistry or collected into a Falcon tube and centrifuged to collect the cell pellet: washed once in PBS, frozen in dry ice and stored at -80°C. A two-way ANOVA test with Bonferronie was performed with a minimum 95% confidence threshold to compare the means of each animal group and the significance was assessed at a value of $p<0.05$.

5.6.4. Lentivirus infection of ependymal cell culture

Cells from *E2f7*$^{flox/flox}$;*E2f8*$^{flox/flox}$ were cultured for ependymal cells as described above. At day 0, *E2f7*$^{flox/flox}$;*E2f8*$^{flox/flox}$ cells were either incubated with GFP- or Cre-coding sequence subcloned into multiple cloning site of a pWPXLD lentivirus plasmid at a multiplicity of infection (MOI) of 2 as described in (Andrusiak, Vandenbosch et al. 2013). The cells were collected 9 days later and fixed for immunocytochemistry. An unpaired two-tailed Student's T-test was performed with a minimum 95% confidence threshold to detect the a difference in the mean at a *P* value of <0.05.

5.6.5. Immunocytochemistry

The medium was aspirated, washed once with PBS and the cells were fixed for 20 minutes at room temperature with 4% PFA. They were then washed 3 times for 5 minutes each with PBS. They were then permeabilized for 10 minutes in 1%Triton/PBS, rinsed once with PBS and blocked 1 hour in 1% donkey serum/PBS. The primary antibodies were diluted in 1% donkey serum/PBS and then incubated at room temperature for 3 hours (Table 5.3). The cells were washed again three times for 5

minutes each with PBS. The appropriate conjugated secondary antibodies were diluted 1:500 in 1% donkey serum/PBS and incubated at room temperature for 1 hour (Table 5.3). The cells were then washed 3 times with PBS and treated with 1mg/mL DAPI at 1:1000 in sterile water for 5 minutes. Then washed two times with PBS and imaged using fluorescent microscope with AxioVision software.

5.7. Western blots

Proteins from neurosphere cultures, ependymal cell culture (day 0, 5 and 10) and P3 SVZ tissue were isolated from cDKO and control animals. Protein was then extracted using a lysis buffer comprised of 10mM Tris pH 7.4, 150mM NaCl, 0.5% Triton and 1mM EDTA, as well as protease inhibitors. Proteins were then quantified by Bradford assay. Western blots were adapted from published protocol (Cregan, MacLaurin et al. 1999). Equal amounts of total protein per sample were electrophoresed through 8 or10% SDS-PAGE, depending on protein size and blotted on a nitrocellulose membrane. Immunoblotting was performed with incubation of primary antibodies overnight at 4°C (Table 5.5). The appropriate conjugated secondary antibodies were incubated for 1 hour at room temperature. Actin was used as loading control. Membranes were incubated with primary antibody Actin (Table 5.5). Blots were developed by chemiluminescence according to manufacturer's instructions (ECL; Amersham Biosciences).

Antibody for Western Blot		
Antibody	**Dilution**	**Source**
	Primary	
PH3 (Rabbit)	1:1000	Millipore
Sox2 (Goat)	1:1000	Santa Cruz
pRb (mouse)	1:1000	BD Biosciences
E2f3 (Rabbit)	1:1000	Santa Cruz
PCNA (Mouse)	1:1000	Millipore
Acetylated Tubulin (Mouse)	1:2000	Sigma-Aldrich
N-Cadherin (Goat)	1:1000	Santa Cruz
E-Cadherin (Goat)	1:1000	Santa Cruz
Numb (Rabbit)	1:1000	Abcam
Actin (Mouse)	1:20 000	Sigma
	Secondary	
HRP conjugated	1:2000	Santa Cruz

Table 5.5: Antibodies used for Western blotting

Antibodies used for this thesis Western blotting are listed, as well as their dilution and the source of the antibody is shown. Secondary antibodies were used depending on the appropriate primary conjugate, the dilution and their sources are also listed.

5.8. Quantitative Real-Time PCR

Total RNA from WT CD-1 differentiation assay (day 0, 1, 3, 5, 7 and 10), neurosphere culture and P3 SVZ tissue from cDKO and control animals and also E12.5 telencephalon tissue from *E2f4*$^{+/+}$ and *E2f4*$^{-/-}$ embryos were extracted using the Trizol method (Invitrogen), according to the manufacturer's instructions. Equal amounts of total RNA per sample were loaded and quantified using a Rotor-Gene RG-3000 (Corbett Research). The SuperScript III Platinum SYBR Green One-Step qRT-PCR (Invitrogen) was used to amplify specific target genes (Table 5.2). All expression values were normalized to GAPDH. An unpaired two-tailed Student's T-test was performed with a minimum 95% confidence threshold to detect the difference in the mean at a *P* value of <0.05, for all graphs in every figure, with the exception of *E2f7* and *E2f8* expression profile (Figure 6.5A-B). *E2f7* and *E2f8* transcript was analysed by a one-way ANOVA and was performed on the means of results followed by a Tukey's post-hoc test. Both tests have a minimum 95% confidence threshold to detect a difference in the mean at a *P* value of <0.05.

5.9. Chromatin Immunoprecipitation

Neurosphere cultures were prepared from WT CD1 embryos at E14.5, as described above for 7 days. ChIP analysis was performed as previously described (Andrusiak, McClellan et al. 2011) in proliferating neurospheres. Antibodies against E2f4 and normal normal rabbit immunoglobulin G (IgG) were obtained from Santa Cruz Biotechnology. The purified DNA was examined by PCR using primers designed around

the E2f consensus site on the *Gli3* promoter (Table 5.2). All results shown are representative from three independent animals.

5.10. Luciferase reporter assay

E2f consensus sites were identified by using Mulan software analysis and ChIP-on-Chip analysis (Julian, Liu et al. 2015). The *Gli3* promoter (1,900 bp) sequence was amplified from mouse genomic DNA by PCR and subcloned into pGL4.24 firefly reporter vector (Promega) (Table 5.2). Sequencing was confirmed by DNA sequencing (StemCore Laboratories, University of Ottawa).

HEK293T cells were transfected using Lipofectamine (Invitrogen) as per manufacturer's protocol. Briefly, one experiment, cells were transfected with pGL4.24 (empty vector), *Gli3* luciferase vector or *Gli3* luciferase vector in combination with E2f4, E2f4 and pRb or E2f4 and p107. In other experiments, cells were transfected with pGL4.24, *Gli3* luciferase vector or *Gli3* luciferase vector in combination with E2f7, E2f8, E2f7 and E2f8, E2f1, E2f1 and E2f7, E2f1 and E2f8 or E2f1, E2f7 and E2f8. The total transfected plasmids were normalized with pcDNA3.1. Transfection efficiency was normalized using internal control pRL Renilla-expressing vector. Cells were lysed at 24-hour post-transfection and examined by spectrophotometer (LMaxII; Molecular Devices) for luciferase expression by a Dual-Glo luciferase kit (Promega) as per manufacturer's protocol. A one-way ANOVA was performed on the means of results from three to four independent experiments followed by a Tukey's post-hoc test to detect a difference in the mean at a P value of <0.05.

5.11. Animal Behaviour

All behavioural procedures were performed according to the University of Ottawa's Behavioural Core facility and Animal Care and Ethics committee and adhered to the guidelines put forth by the Canadian Council on Animal Care. Age-matched cDKO and control mice were maintained on FVBN background. One cohort of male and female mice, age 8-9 weeks at the onset of behavioural testing, with the exception of ultrasonic vocalization, age P8-P10, were blindly tested.

5.11.1. Juvenile Interaction

Juvenile interaction testing was adapted from published protocol (Crawley 2004) and described in (Tabuchi, Blundell et al. 2007). This behavioural test is conducted under a red light. Briefly, the animals are habituated in the test room for 15 minutes. Simultaneously, the test mouse is placed in a new cage with an unfamiliar juvenile (around P21) of the same gender and strain. They are permitted to interact for 2 minutes. The total time the test mouse is interacting with the juvenile is recorded using a manual stopwatch. Three days later, the procedure is repeated with the same juvenile mouse and the test mouse. A two-way ANOVA test with Bonferronie was performed with a minimum 95% confidence threshold to compare the means of each animal group and the significance was assessed at a value of $p<0.05$.

5.11.2. Adult Social Interaction

Adult social interaction testing was adapted and described from published protocol (Crawley 2004). This behavioural test is conducted under a red light. Briefly,

the animals are habituated in the test room for 15 minutes. The test mouse is placed in the corner of an open field box (45cm x 45cm) that contains an empty wire mesh rectangular cage (5.5cm x 9,6cm) and left to explore for a total time of 5 minutes. The test mouse is then removed and a social target mouse is placed in the wire mesh caged (an unfamiliar mouse of same gender and strain). The test mouse is then placed back into the corner of the open field box and left to explore for another 5 minutes. The time spent by the test mouse interacting with the zone surrounding the social target mouse is recorded using Ethovision software from Noldus. A two-way ANOVA test with Bonferronie was performed with a minimum 95% confidence threshold to compare the means of each animal group and the significance was assessed at a value of $p<0.05$.

5.11.3. Olfactory Habituation-Dishabituation

Olfactory habituation-dishabituation testing was adapted from published protocol (Crawley 2004) and described in (Crawley, Chen et al. 2007). This behavioural test is conducted in normal level lighting. Briefly, the animals are habituated in the test room for 30 minutes. A dry applicator is inserted in the water bottle hole and the test mouse is then habituated for another 30 minutes, this step helps decrease neophobia to the applicator tip. The first non-social odour is water (applicator dipped in water). The applicator is then inserted into the water bottle hole of the cage and the time the target mouse interacts with the tip of the application is reordered using a manual stopwatch. Every trial is a total of 2 minutes. A new applicator dipped in water will be presented two more times. The almond extract is the second non-social odour, while banana extract is the third (1:100 dilution of extracts). The first social odour is from an unfamiliar male

FVBN mouse, while the second social odour is from an unfamiliar female FVBN mouse. A two-way ANOVA test with Bonferronie was performed with a minimum 95% confidence threshold to compare the means of each animal group and the significance was assessed at a value of $p<0.05$.

5.11.4. Ultrasonic Vocalization

Ultrasonic vocalization testing was adapted from published protocol (Crawley 2004) and described in (Shu, Cho et al. 2005). This behavioural test is conducted in normal level lighting. Briefly, the pups and their dame were acclimatized to the testing room 1 week prior to testing and pups were handled 2 times per day during the three days leading up to testing. One at the time, P8 pups are placed into the testing box, which contains 4 ultrasound detectors that can each detect a specific frequency (50, 60, 70 and 80Hz). Test animals are recorded for 5 minutes and then returned to the litter with the dame. The same test animals are tested 2 days later in the same fashion. The number of ultrasonic events and duration of the vocalization is recorded using the Ultravox system and technology. A two-way ANOVA test with Bonferronie was performed with a minimum 95% confidence threshold to compare the means of each animal group and the significance was assessed at a value of $p<0.05$.

5.11.5. Nest Building

Nesting behaviour testing was adapted from published protocol (Deacon 2012). Test animals are individually housed prior to testing. The test cage contains normal bedding and one Nestlet, which is a standard 5cm square, pressed cotton batting

(provided by the University of Ottawa ACVS). The test animal is placed in the test cage approximately 1 hour before the dark cycle. The results are assessed the next morning. The quality of the nest is scored using a 5 point systems described in (Deacon 2012). An unpaired two-tailed Student's T-test was performed with a minimum 95% confidence threshold to detect a difference in the mean at a *P* value of <0.05.

5.11.6. Prepulse Inhibition Test

Prepulse inhibition test was performed as previously described (Liu, Hong et al. 2013). This behavioural test is conducted in a startle chamber, which is capable of detecting whole body flinch and these flinches are recorded in millivolts. The prepulse effect was measured using varying prepulse intensity on prepulse inhibition (PPI). Briefly, for startle habituation and environment acclimatization, the animals are placed in a plexiglass cylinder for 5 minutes. Background noise was maintained at 65dB. After acclimatization, the pulse (120dB tone, 50ms) was presented alone or in the presence of a prepulse tone of 4, 8 or 16dB above the background with a fixed interval of 100ms prior to pulse. The experiment contains 4 blocks of 6 trials organized in a pseudo-random order within the blocks. The response to the 120dB in the presence of the prepulse (4, 8 or 16dB above the background noise) is the PPI. The percentage of PPI for each animal is calculated using the following formula: %PPI=100x[(pulse alone score)-(prepulse +pulse score)]/(pulse alone score), where the score is the amplitude of the response. The SR-Lab Startle response System is used in this protocol. A two-way ANOVA test with Bonferronie was performed with a minimum 95% confidence threshold to compare the means of each animal group and the significance was assessed at a value of $p<0.05$.

6. RESULTS

6.1. Generation of CNS-specific *E2f7* and *E2f8* double knockout

Little is known regarding the functional role of atypical E2fs in CNS development. Because whole-embryo combined deletion of *E2f7* and *E2f8* results in placental defects and mid-gestation lethality, around E11.5 (Li, Ran et al. 2008), it has not been possible to determine the role of atypical E2fs in CNS development, which occurs predominantly during mid to late gestation. To solve this problem, we began by generating a novel mouse model in which deletion of the atypical E2fs was specific to the CNS. Mice bearing floxed alleles for *E2f7* and *E2f8* (Li, Ran et al. 2008) were interbred with mice expressing *Cre* recombinase in neural stem and progenitor cells by expressing Cre under the control of the *nestin* promoter (Berube, Mangelsdorf et al. 2005). In mice, nestin is expressed as early as embryonic day 8.0 (E8.0), in the cerebellum and the telencephalon (Tronche, Kellendonk et al. 1999), and using this *Cre* driver recombination of the floxed allele occurs between E8.5-E9.5 (Berube, Mangelsdorf et al. 2005).

We confirmed efficient *Cre*-mediated recombination of the floxed *E2F7* and *E2F8* alleles using quantitative real-time PCR (qRT-PCR). qRT-PCR analysis of SVZ tissue RNA, isolated from 6-month old cDKO mice, revealed a 95% reduction of *E2f7* and *E2f8* mRNA (Figure 6.1). Furthermore, *E2f7* and *E2f8* mRNAs were almost undetectable in embryonic neural precursor cells cultured from cDKO embryos, while the known atypical E2f target gene, *Cdc6*, was upregulated (Li, Ran et al. 2008) (Figure 6.1).

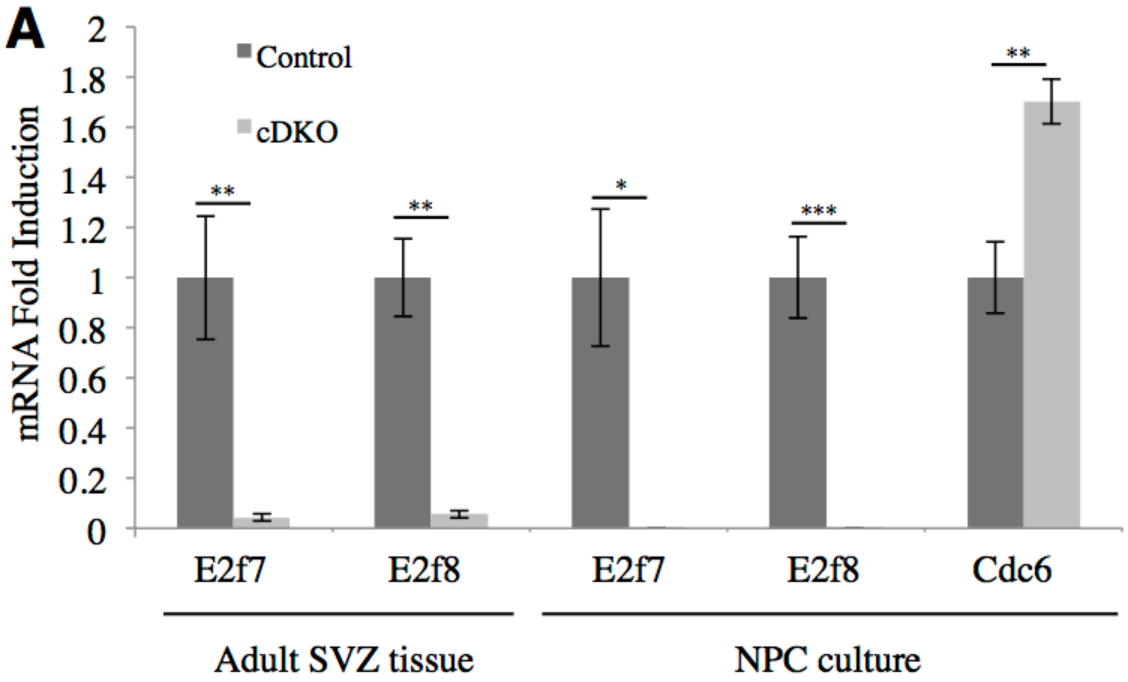


Figure 6.1: *E2f7* and *E2f8* expression results in efficient recombination of floxed alleles

Quantitative real-time PCR analysis of *E2f7* and *E2f8* expression in 6-month old cortical tissue and *E2f7*, *E2f8* and *Cdc6* expression in cultured neural precursor cells (NPC) with the indicated genotype. Samples are normalized to loading control GAPDH and expressed as fold induction over control (n=4).
Error bars represent SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

To assess the survival rate of these conditional mutants, mice homozygous for the *E2f7* and *E2f8* floxed alleles [$E2f7^{flox/flox};E2f8^{flox/flox}$] were interbred with double heterozygous mice [$Nestin\text{-}Cre;\ E2f7^{flox/+};E2f8^{flox/+}$] (Table 5.1). *E2f7*/*E2f8* conditional knockout (cDKO), cE2f7 and cE2f8 mice were born at the expected Mendalian ratio (Table 6.1A), indicating loss of both *E2f7* and *E2f8* did not increase the rate of morbidity or mortality in these litters. All progeny appeared normal in size, displayed no gross anatomical defects by 6 months of age, and were fertile. Thus, atypical E2fs are not required for postnatal viability.

Taken together, these results confirm efficient *Cre*-mediated recombination of the *E2f7* and *E2f8* floxed alleles, which results in loss of *E2f7* and *E2f8* mRNA in the adult SVZ and embryonic cultured neural precursor cells. Thus, we have created a model system allowing us to study the role of atypical E2fs in telencephalic development.

A *Nestin-Cre;E2f7$^{flox/+}$;E2f8$^{flox/+}$* x *E2f7$^{flox/flox}$;E2f8$^{flox/flox}$*					
E2f7	flox/+ or flox/flox	flox/+	flox/flox	flox/+	flox/flox
E2f8	flox/+or flox/flox	flox/+	flox/+	flox/flox	flox/+
Nestin-Cre	-	+	+	+	+
Number	25	5	7	7	11
Percent	45.5%	9.1%	12.7%	12.7%	20%
Expected percent	50%	12.5%	12.5%	12.5%	12.5%

B *Nestin-Cre;E2f7$^{flox/flox}$;E2f8$^{flox/flox}$ E2f1$^{+/-}$;E2f3$^{flox/+}$* x *E2f7$^{flox/flox}$;E2f8$^{flox/flox}$ E2f1$^{+/-}$;E2f3$^{flox/+}$*										
E2f7	flox/ flox	flox/ flox	flox/ flox	flox/ flox	flox/ flox	flox/ flox	flox/ flox	flox/ flox	flox/ flox	flox/ flox
E2f8	flox/ flox	flox/ flox	flox/ flox	flox/ flox	flox/ flox	flox/ flox	flox/ flox	flox/ flox	flox/ flox	flox/ flox
E2f1	+/+, +/- or -/-	+/+	+/+	+/+	+/-	-/-	-/+	-/-	+/-	-/-
E2f3	+/+, flox/+ or flox/ flox	+/+	flox/+	flox/ flox	+/+	+/+	flox/+	flox/+	flox/ flox	flox/ flox
Nestin-Cre	-	+	+	+	+	+	+	+	+	+
Numbers	22	3	2	1	1	1	7	5	6	0
Percent	45.8%	6.3%	4.4%	2.1%	2.1%	2.1%	14.6%	10.4%	12.5%	0
Expected percent	50%	3.1%	6.3%	3.1%	6.3%	3.1%	12.5%	6.3%	6.3%	3.1%

Table 6.1: Genotype frequency of mouse cross progeny

Parental genotypes are indicated in (**A-B**). Expected percent assumes Mendelian ratio for all genotypes.

6.2. Loss of atypical E2fs leads to enlargement of the lateral ventricle

To determine the role of atypical E2Fs in the telencephalon, gross neural anatomy was examined at 6 months using cresyl violet staining in the following animals: no-*Cre* and Cre-controls, cE2f7, cE2f8 and cDKO. Given that high nuclear expression of *Cre* recombinase was shown to have potential toxic effects and compromise brain development (Forni, Scuoppo et al. 2006), we first analyzed no-Cre and Cre-expressing controls for potential Cre-mediated toxic effects. No changes in the anatomical structures could be detected in Cre-expressing brains compared to no-Cre brain, indicating that both genotypes could be used as controls (Figure 6.2A).

When examining the *E2f7* and *E2f8* deficient brains, we found that the lateral ventricles (LV) were enlarged in the cE2f7 and cDKO mutants, while the cE2f8 was similar to the no-Cre and Cre controls (Figure 6.2A). Measurement of the area of the LV revealed a 7.5- to 10-fold increase in cE2f7 and cDKO brains (Figure 6.2B). Furthermore, sagittal sections demonstrated a pronounced rostro-caudal enlargement of the LV in the cDKO mice (Figure 6.2C), suggesting loss of *E2f7* leads to ventriculomegaly. To avoid potential compensation for the loss of *E2f7* by *E2f8*, all subsequent experiments were performed with no-Cre-control (controls) and cDKO animals.

To investigate the timing of the onset of ventriculomegaly in the cDKO mice, we performed a time course to determine the age at which LV enlargement becomes evident. Anatomical and histological analysis of brains stained by cresyl violet from late gestation (E18.5) revealed no difference in LV size, however, a 4-fold increase in the area of the

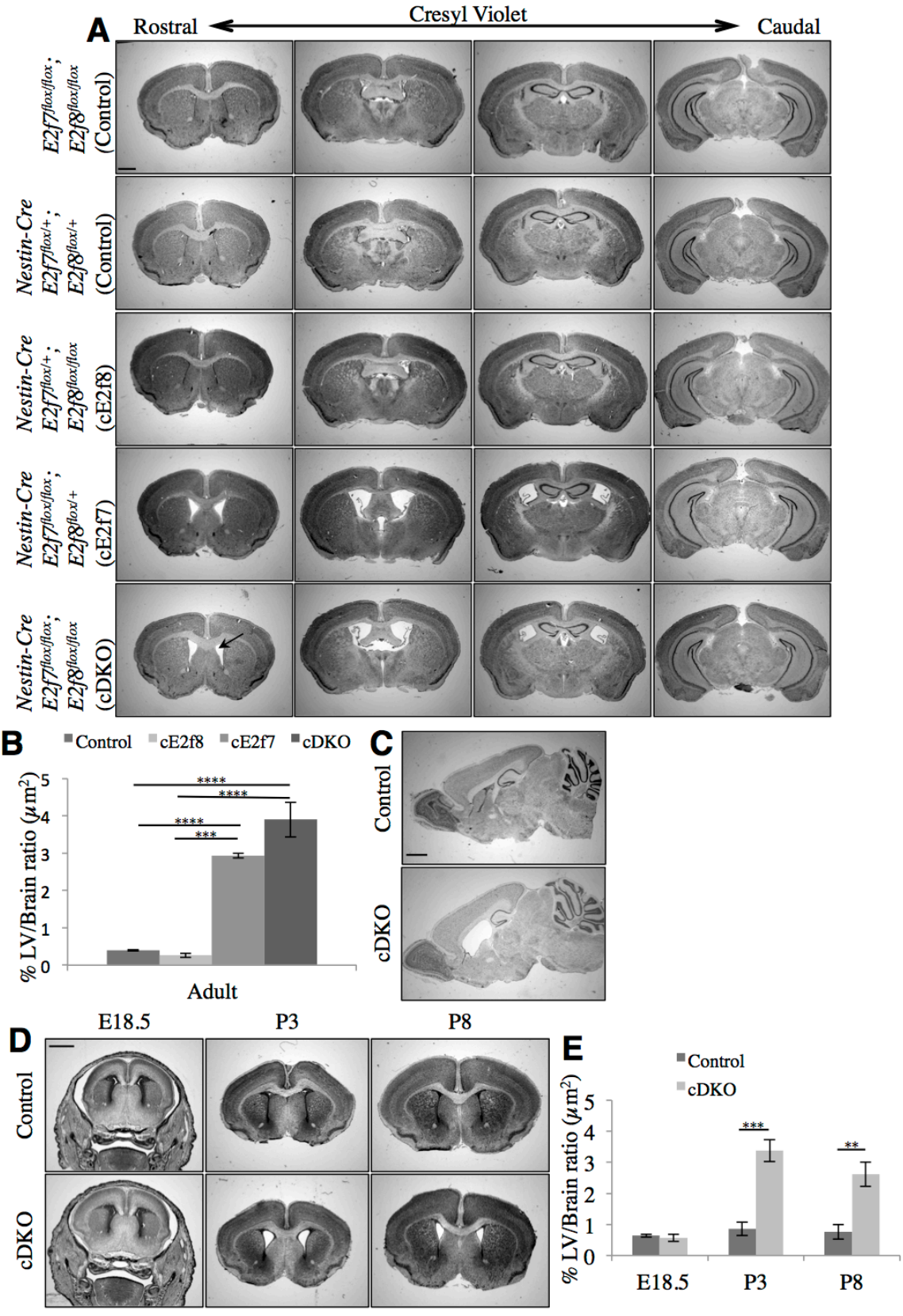

A
Rostral
Cresyl Violet
Caudal
E2f7flox/flox; E2f8flox/flox (Control)
Nestin-Cre E2f7flox/+; E2f8flox/+ (Control)
Nestin-Cre E2f7flox/+; E2f8flox/flox (cE2f8)
Nestin-Cre E2f7flox/flox; E2f8flox/+ (cE2f7)
Nestin-Cre E2f7flox/flox; E2f8flox/flox (cDKO)
B
Control
cE2f8
cE2f7
cDKO
% LV/Brain ratio (μm2)
Adult
C
Control
cDKO
D
E18.5
P3
P8
Control
cDKO
E
Control
cDKO
% LV/Brain ratio (μm2)
E18.5
P3
P8

Figure 6.2

Figure 6.2: Expansion of the lateral ventricle in cDKO and cE2f7 mice.

(**A**) Cresyl violet staining of brain coronal sections from 6-month adult mice of the indicated genotypes. (Arrow indicates lateral ventricle (LV)) (**B**) The area of both right and left LV was measured and divided by the total area of the brain (%LV/Brain ratio) of the indicated genotypes (n=6 control, n=3 cE2f7, n=3 cE2f8, n=4 cDKO), no significant differences between the ratio between the 7 weeks and 24 week mice, animals were binned together. (**C**) Representative cresyl violet staining of sagittal section of no-Cre control (n=3) and cDKO (n=3) 6 month adult mice. (**D**) Coronal section of cresyl violet staining demonstrate enlargement of the lateral ventricle at P3, P8 but not at E18.5. (**E**) %LV/brain ratio analysis of the ventricles at E18.5 (n= 3 control, n=4 cDKO), P3 (n=4) and P8 (n= 4 no-cre control, n=3 cDKO). (**B**) One-way ANOVA with Tukey's was performed to compare the %LV/Brain ratio, (**E**) Student T-test was performed relative to control. Scale bars 1 mm. Error bars represent SEM. $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

LV was found at P3 and 5 days later at P8 (Figure 6.2D-E). These results demonstrate loss of atypical E2fs consistently leads to a significant increase in postnatal ventricular size (ventriculomegaly).

Ventriculomegaly is associated with many human developmental disorders including idiopathic mental retardation, autism spectrum disorder (ASD) and schizophrenia (Gilmore, van Tol et al. 2001). Ventriculomegaly may be caused by a wide variety of developmental abnormalities which include obstruction of the aqueduct system (Rekate 2008, Huh, Todd et al. 2009), abnormal proliferation of embryonic neural precursor cells (Takahashi, Nowakowski et al. 1994, Caviness, Takahashi et al. 1995, Takahashi, Nowakowski et al. 1995, Chae, Kim et al. 2004), disruption in neuronal differentiation (Chae, Kim et al. 2004, Paez, Batiz et al. 2007), cortical apoptosis (Medina-Bolivar, Gonzalez-Arnay et al. 2014, Park, Lee et al. 2015), and disruption of the ependymal cell barrier (Dominguez-Pinos, Paez et al. 2005, Shook, Lennington et al. 2014). In the following sections, I will explore these possibilities to determine the underlying defect leading to ventriculomegaly in cDKO mutants.

6.3. Enlargement of the LV not due to obstruction of the sylvian aqueduct or thinning of the subcommissural organ

Congenital ventriculomegaly is frequently associated with the collapse of the cerebral aqueduct, also known as sylvian aqueduct or, malformation and thinning of the subcommissural organ (SCO), a secretory gland situated above the sylvian aqueduct (Casey, Kimmings et al. 1997, Huh, Todd et al. 2009, Rodriguez, Guerra et al. 2012). As

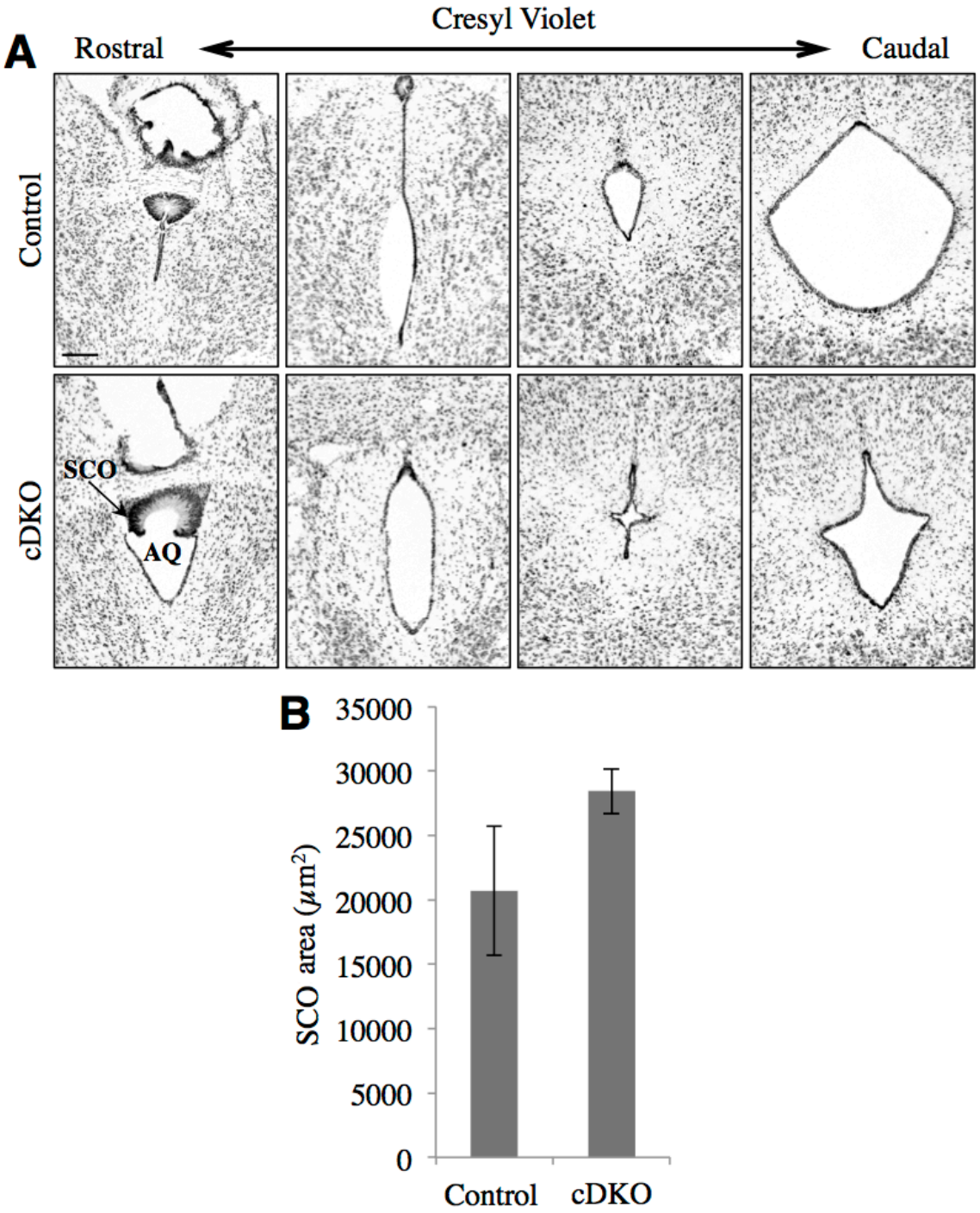


Figure 6.3: Aqueduct collapse is not observed in cDKO mutants

(**A**) Cresyl violet staining of PFA coronal sections of sylvian aqueduct (AQ) and arrow represent subcommissural organ (SCO) of control and cDKO 6-month-old mice. (**B**) Quantification of the SCO area between control (n=3) and cDKO (n=3). Student T-test was performed relative to control. Scale bar 100 μm. Error bars represent SEM.

the sylvian aqueduct connects the third and fourth ventricles and is located in the mesencephalon (Huh, Todd et al. 2009), restriction or blockage of the flow of the CSF leads to increased ventricular pressure and dilatation of the ventricular system (Perez-Figares, Jimenez et al. 2001). To determine if the collapse of the sylvian aqueduct is the cause of ventriculomegaly in the cDKO animals, cresyl violet staining was used to evaluate the anatomical structure in control and cDKO animals (Figure 6.3A). No collapse of the sylvian aqueduct was observed. In addition, the SCO size was similar between genotypes, with no evidence of malformation or thinning between our mutant and control littermates (Figure 6.3A-B). Together, these results suggest that the primary cause of ventriculomegaly in the compound mutants is not due to a systemic accumulation of CSF or due to a collapse of the sylvian aqueduct or thinning of the SCO.

6.4. The role of atypical E2fs in neural stem cell regulation

Overproduction of neurons at the expense of neural precursor cells can lead to enlargement of the LV and consequently loss of the VZ (Takahashi, Nowakowski et al. 1994, Caviness, Takahashi et al. 1995, Takahashi, Nowakowski et al. 1995, Chae, Kim et al. 2004). Previous evidence suggests that components of the Rb/E2f pathway play a key role in regulating the pool of neural precursors cells (Ruzhynsky, McClellan et al. 2007, Vanderluit, Wylie et al. 2007, McClellan, Vanderluit et al. 2009, Julian, Vandenbosch et al. 2013). Given the importance of atypical E2fs in mediating a cell-cycle-dependent feedback loop with members of the E2f family, we asked whether atypical E2fs might be regulating components of the canonical Rb/E2f pathway. To address this question, we examined the expression of members of the Rb/E2fs family by Western blotting in E14.5

ganglionic eminence (GE)-cultured neurospheres in control and cDKO brains. We detected increased expression of pRb, E2f3a, and the E2f3 target gene, Sox2, protein levels in the cDKO neurospheres. In contrast, no changes in expression levels of E2f3b or the pRb downstream target, proliferating cell nuclear antigen (PCNA) were detected (Figure 6.4A). Since it has previously been demonstrated that loss of E2f3a increases the number of primary neurospheres (Julian, Vandenbosch et al. 2013), we questioned whether loss of atypical E2fs depletes the neural precursor population. We performed a primary neurosphere assay (Reynolds and Weiss 1992), on isolated neural precursor cells at E14.5 from the GE of control and cDKO brains. Cells from the GEs were dissociated into a single-cell suspension, plated at clonal density, and grown for 7 days. Quantification of neurosphere numbers revealed no significant differences between cDKO and control cultures (Figure 6.4B). Although pRb and E2f3 are involved in regulating the pool of neural precursor cells (Ferguson, Vanderluit et al. 2002, Julian, Vandenbosch et al. 2013), we detected no changes in the number of neurosphere-forming cells in the cDKO embryonic brain.

Considering cDKO neurospheres have enhanced pRb, E2f3a and Sox2, and it has been previously shown that E2f3a regulates *Sox2* expression in apical neural precursors cells *in vivo* (Julian, Vandenbosch et al. 2013), we asked whether loss of atypical E2fs could impact the neural precursor cell population. Using immunohistochemistry, we stained E14.5 cerebral cortical tissue with markers for apical precursor (Sox2) cells, residing in the VZ and contain the neural stem cell population, basal progenitor (Tbr2) cells and phosphohistone H3 (PH3), which labels cells in M-phase (Figure 6. 4C&E).

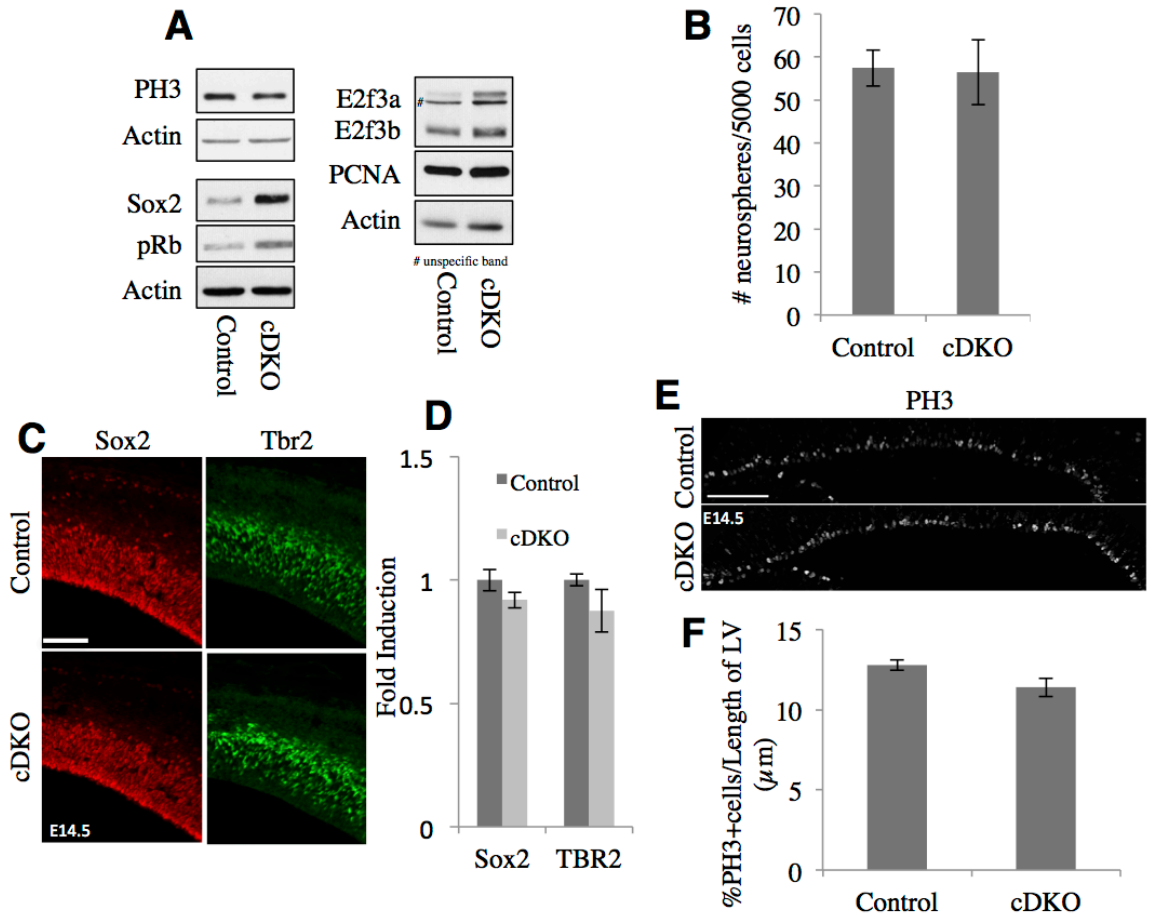


Figure 6.4: The role of atypical E2fs in regulation of neural precursor cell proliferation

(**A**) Western blot analysis was performed on neural precursor cells isolated from E14.5 cDKO and control embryos. *E2f7* and *E2f8* conditional double mutant (n=3) showed down regulation of PH3 and upregulation of Sox2, pRb and E2f3a compared to levels in the control (n=3). (**B**) cDKO (n=4) mutant generated the same numbers of primary neurospheres derived from E14.5 GE then the control (n=7). (**C-D**) Coronal sections of E14.5 control (n=4) and cDKO (n=4) embryo were immunostained with Sox2 and Tbr2 and quantification of $Sox2^{+}$ and $Tbr2^{+}$ cells within the dorsal cortex is represented as a fold induction relative to control. (**E-F**) Proliferation was assessed by immunostaining for PH3 cells at E14.5. Quantification represents the number of PH3 cells lining the VZ normalized to the length of the LV (n=4). Student T-test was performed relative to control. Scale bars 100 μm. Error bars represent SEM.

Quantification of the number of $Sox2^+$ apical precursor cells and $Tbr2^+$ committed basal progenitors revealed no changes between the control and the compound mutants (Figure 6.4D). We also observed no changes in the number of proliferating cells in M-phase lining the wall of the LV (Figure 6.4E-F).

These results indicate that, in spite of the increase of pRb and E2f3a protein levels in cDKO neurospheres, we could not detect effects of atypical E2fs deficiency on the embryonic neural precursor cell population. Therefore, we asked whether ventriculomgelay in the cDKO mice is a result of the deregulation of the neural differentiation machinery rather then a depletion of the pool of neural precursor cells.

6.5. The role of atypical E2fs in neural differentiation and commitment

Considering that telencephalon specific deletion of *Rb1* enhances E2f7 activity (Ghanem, Andrusiak et al. 2012), and that E2f7 can directly repress transcription of *Dlx1* and *Dlx2* genes involved in neuronal differentiation (Ghanem, Andrusiak et al. 2012), we investigated whether atypical E2fs regulate neuronal differentiation. We first asked if atypical E2fs were differentially expressed during *in vitro* differentiation of NPCs to generate neurons and glial cells. To address this question, we used qRT-PCR to analyze *E2f7* and *E2f8* transcript expression at different stages of wild type (WT) neural differentiation. *E2f7* mRNA expression significantly peaked at 3 days of differentiation (D3) with a 5-fold increase compared to undifferentiated WT neurospheres (D0), and returned to basal expression levels at 5-days post-differentiation (D5) (Figure 6.5A). In contrast, *E2f8* mRNA expression remained constant during neural differentiation (Figure 6.5B). Since the peak in *E2f7* transcript expression occurred at D3, which corresponds to

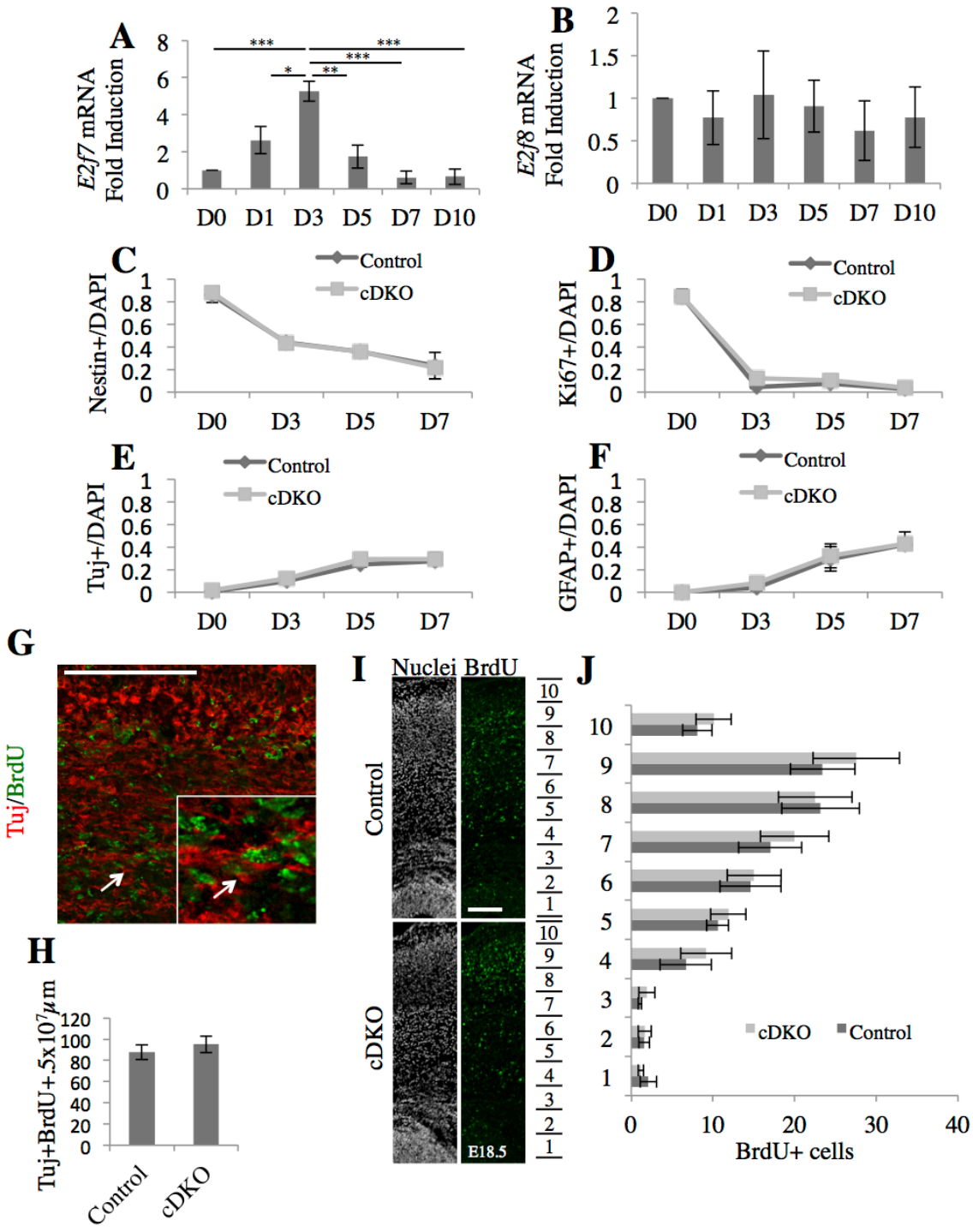

A
E2f7 mRNA Fold Induction
D0 D1 D3 D5 D7 D10
B
E2f8 mRNA Fold Induction
C
Nestin+/DAPI
Control
cDKO
D
Ki67+/DAPI
E
Tuj+/DAPI
F
GFAP+/DAPI
G
Tuj/BrdU
H
Tuj+BrdU+.5x10^7μm
Control
cDKO
I
Nuclei BrdU
E18.5
J
BrdU+ cells

Figure 6.5

Figure 6.5: The role of atypical E2fs in neuronal commitment and differentiation *in vitro* and *in vivo*

Quantitative real-time PCR analysis of *E2f7* (**A**) and *E2f8* (**B**) expression of wild-type (WT) E14.5 differentiated neural precursor cells over 10 days. Samples are normalized to loading control GAPDH and fold induction is represented by baseline day 0 (n=3). (**C-F**) NPCs (nestin), neurons (Tuj), astrocytes (GFAP) and marker of proliferation (Ki67) in differentiating neurospheres derived from telencephalic neuroepithelia of E14.5 control and cDKO mouse embryos. Days 0, 3, 5 and 7 *in vitro* in differentiating medium with FBS shows no difference between control and cDKO cultures. Results represented as total DAPI proportion (n=4). (**G-H**) BrdU and Tuj staining in E14.5 coronal sections following a 24hrs BrdU pulse to identify cells that have exited the cell cycle. The number of cells expressing both BrdU and Tuj was quantified with a defined area of the SVZ (5 x 10^7μm) (arrow shows example of quantified cells) (n=4). (**I-J**) Long-term BrdU birthdating analysis (E13.5-E18.5) where pregnant mice were injected at gestational day E13.5 with BrdU and embryos were taken at E18.5. Embryos were collected at E18.5, stained with BrdU and $BrdU^+$ cells were counted in 10 equal arbitrary layers of the cortex to systematically investigate the difference between control (n=4) and cDKO (n=4) embryos. (**A-B**) One-way ANOVA with Tukey's was performed to compare mRNA transcript. (**C-F&J**) Two-way ANOVA with Bonferroni was performed for multiple comparisons (**H**) Student T-test was performed relative to control. Scale bar 1 mm. Scale bars 100 μm. Error bars represent SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

the beginning of neuronal differentiation, we next assessed the ability of NPCs isolated from cDKO neurospheres to differentiate in vitro. NPCs from both control and cDKO were assessed after 7 days *in vitro* differentiation by immunocyctochemistry with markers for neurons (βIII-tubulin -Tuj1), astrocytes (glial fibrillary acidic protein (GFAP), neural precursor cells (nestin) and proliferation (Ki67). The percentage of nestin-positive cells showed a progressive decrease during the course of differentiation (Figure 6.5C), while Ki67 also showed a rapid decrease after D0 in both genotypes (Figure 6.5D). In contrast, the percentage of Tuj- and GFAP-expressing cells increased progressively during the course of differentiation in both the control and cDKO differentiated cultures (Figure 6.5E-F). In summary, no difference was detected between cDKO and the controls in their ability to proliferate and generate neurons and astrocytes in culture.

Although we saw no differences in neural differentiation in the compound mutants *in vitro*, we questioned whether the cortical environment was altered *in vivo*. Animal models of ventriculomegly have shown excessive production of neurons in the cortex leading to sever impairment in embryonic neurogenesis (Chae, Kim et al. 2004, Paez, Batiz et al. 2007). To determine if loss of atypical E2fs impacts neuronal fate decision, we performed a neuronal commitment assay. Pregnant mice were injected at E13.5 with a single dose of bromodeoxyuridine (BrdU), which is taken up by neural precursor cells during S-phase. To visualize BrdU containing cells that have exited the cell cycle and initiated differentiation, animals were sacrificed 24h later and newly committed cells that have undergone terminal mitosis and have migrated out of the SVZ were identified by double labeling with BrdU and Tuj1 (Figure 6.5G). Quantitation of

the double-labeled cells in cDKO mice and littermate controls revealed no significant changes in the number of newly committed cells in the developing cortex (Figure 6.5H).

To determine whether neurons from the compound mutants were born at the appropriate developmental time, we performed a neuronal birthdating assay. Pregnant mice were injected with a single dose of BrdU at E13.5, a peak period of neurogenesis, corresponding to the time where deep layer cortical neurons are generated. Embryos were collected and coronally sectioned 5 days later at E18.5, which marks the end of cortical neurogenesis. This technique permits quantitative analysis of neurons which are undergoing terminal mitosis at the time of BrdU injection and then go on to migrate to their ultimate destination in the cerebral cortex. For quantification, the cortex was divided into 10 equal sections, spanning from the VZ to the CP, and within each section, $BrdU^+$ cells were counted (Figure 6.5I-J). As compared to the controls, cDKO mice showed no difference in the number of neurons born at E13.5, suggesting that similar numbers of neurons are born during peak of neurogenesis in the developing cortex of the cDKO and the control embryos.

Taken together, our results demonstrate that neural precursor cells have the ability to proliferate and differentiate in the absence of atypical E2fs. Furthermore, atypical E2fs do not appear to regulate embryonic neuronal commitment and, neurons are born at the appropriate times in compound mutant mice. Overall, these findings strongly suggest that ventriculomegaly observed in the cDKO mice is not due to excessive neuronal production during embryonic neurogenesis.

6.6 Loss of atypical E2fs results in postnatal apoptosis

Whole embryo knockouts for atypical E2fs, are characterized by widespread apoptosis throughout the CNS and die by E11.5 from marked placental defects (Li, Ran et al. 2008). Although, *E2f7*$^{-/-}$*:E2f8*$^{-/-}$ embryos with WT-placentas die at birth, they still displayed pronounced apoptosis (Ouseph, Li et al. 2012). Since it has been previously shown that cell death may contribute to morphological changes in the brain leading to ventriculomegaly (Park, Lee et al. 2015), we asked whether cell death was present in the telencephalon of the compound mutants. We examined the levels of apoptosis before (E14.5) and after (P8) the onset of ventriculomegaly. We analyzed the *E2f7/E2f8* mutants at E14.5 and P8 for evidence of activated caspase-3 (AC-3). At E14.5, we observed no increase in AC3^{+} apoptotic cells in the mutants' telencephalon. However, at P8, we detect a 1.5-fold increase in AC3^{+} cells in the telencephalon (Figure 6.6A-B). Ventriculomegaly animal models are often associated with damage and disruption of the SVZ neural stem cell niche (Jimenez, Tome et al. 2001, Wagner, Batiz et al. 2003, Paez, Batiz et al. 2007). The SVZ neural stem cell niche is situated along the lateral wall of the lateral ventricle. We therefore, quantified the number of apoptotic cells within this region. Our result revealed no changes at E14.5 and a 1.5-fold increase of AC3^{+} cells in the VZ/SVZ of the cDKO at P8 (Figure 6.6A-B). These results suggest inappropriate postnatal cell death. Increase cell death in the compound mutant postnatal VZ/SVZ suggests that newly committed neurons maybe be dying in the SVZ.

To determine if an increase in cell death in the VZ/SVZ was responsible for ventriculomegaly in the *E2f7/E2f8* mutants' brains due to neuronal loss, we questioned whether atypical E2fs are required for the survival of adult born neurons. We inactivated

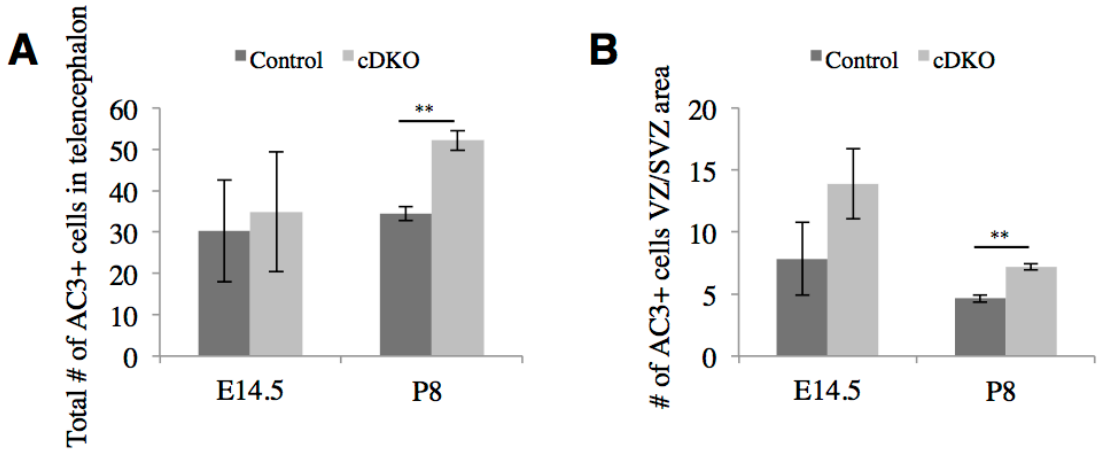


Figure 6.6: Increased cell death in postnatal cDKO animals

To determine whether apoptosis was present before and after the onset of ventriculomegaly in the compound mutants, we counted the number of apoptotic cells at E14.5 and P8. Apoptotic cells were identified by active caspase-3 immunohistochemistry. (**A**) Total apoptotic cells were counted across 5 representative sections in both left and right hemisphere through the telencephalon. (**B**) Apoptotic cells were counted within the VZ/SVZ area, delineated by DAPI nuclear staining as described in (**A**). Cell counts revealed an increase in apoptotic cells in the telencephalon and VZ/SVZ of the cDKO mice at P8 (Control, n=3; cDKO, n=3), but not in the developing cortex at E14.5 embryos (Control, n=4; cDKO, n=4). Student T-test was performed relative to control. Error bars represent SEM. $^{**}p<0.01$.

atypical E2fs in transit-amplifying progenitors *in vivo* using a Cre-expressing retrovirus targeting only cells in M-phase, which give rise to newborn neurons in the SVZ and dentate gyrus (DG) of the hippocampus, which are the two neurogenic niches in the adult brain. Nine-week old *E2f7*$^{flox/flox}$*;E2f8*$^{flox/flox}$ (*E2f7;E2f8* DKO) and *E2f7*$^{flox/+}$*;E2f8*$^{flox/+}$ (*E2f7;E2f8* Control) animals were stereotaxically injected in the SVZ and DG with a mixture of retrovirus vector expressing both i) *Cre* recombinase: green fluorescence protein (GFP); and also ii) an internal control vector carrying red florescence protein (RFP) (Figure 6.7A). The animals were sacrificed 28 days post injection (dpi) and the survival of newborn neurons was quantified and expressed as the number of double-labeled Cre-GFP^{+}/RFP^{+} cells normalized to the total number of RFP^{+} cells. This retroviral-mediated knockout technique enables us to determine the survival capacity of newborn neurons without being influenced by the total number of infected cells. In the DG, the fraction of double-labeled cells over total RFP-labeled cells reveals no differences between *E2f7;E2f8* DKO and *E2f7:E2f8* control animals at 28dpi (Figure 6.7B). In the SVZ, newborn neurons migrate tangentially along the RMS and by 28dpi have incorporated into the olfactory bulb (OB). Similarly to the results observed in the DG, the fraction of double-labeled cells in the OB did not differ between genotypes (Figure 6.6B). These results indicates that acute knockout of atypical E2fs in fast proliferating neural precursor adult SVZ and DG cells can still survive and migrate to their appropriate destination. Adult newborn neurons do not appear to be affected by the absence of atypical E2fs. An alternative explanation may be incomplete *Cre*-mediated excision, since immunostaining-quality antibodies for atypical E2fs are currently not efficient for mouse, we could not assess *Cre*-specific deletion of *E2f7* and *E2f8*,

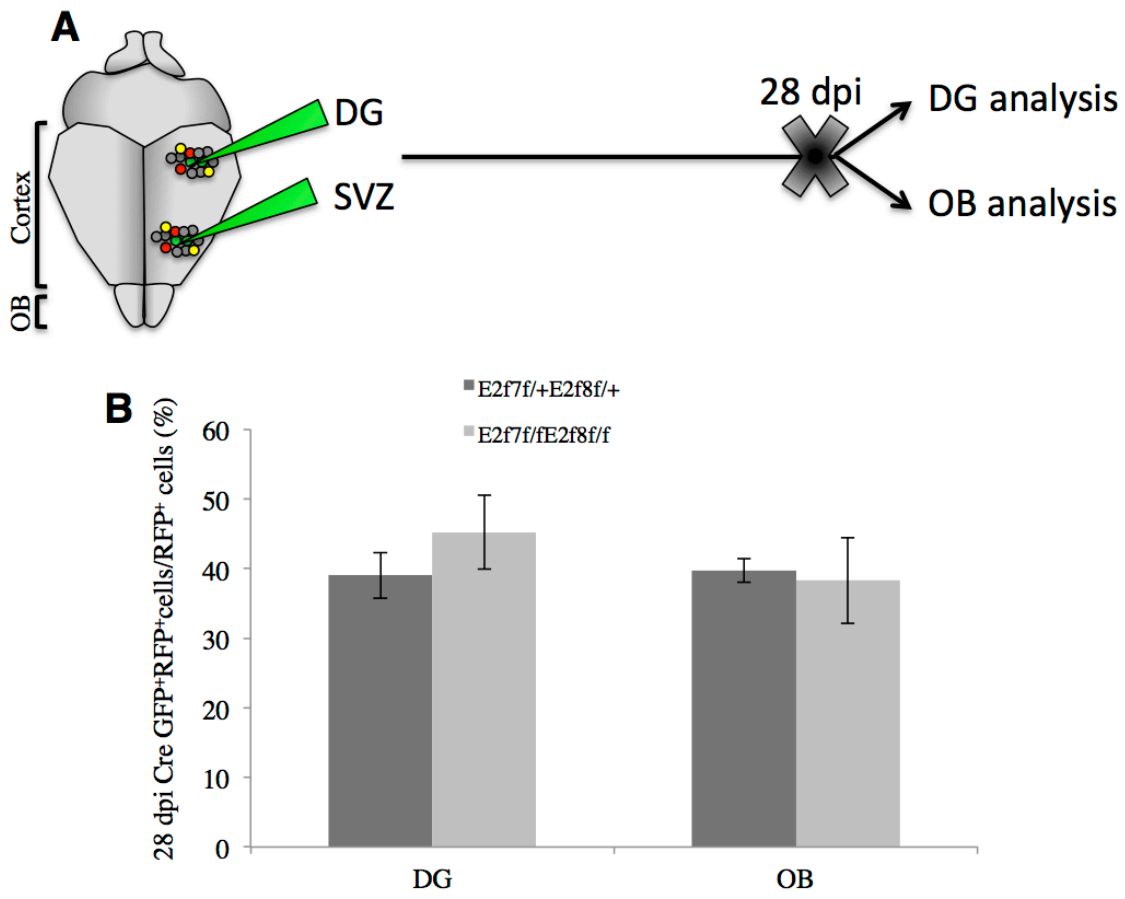


Figure 6.7: Single-cell deletion of *E2f7* and *E2f8* shows normal neuronal survival in the DG and the SVZ

(**A**) Diagram depicting the experimental paradigm. Single-cell deletion of *E2f7* and *E2f8* was performed by viral injections of vector expressing both Cre recombinase and green fluorescence protein (GFP) co-injected with internal control vector red florescence protein (RFP) in the DG and SVZ of control $E2f7^{flox/+};E2f8^{flox/+}$ and mutant $E2f7^{flox/flox};E2f8^{flox/flox}$ 8 week old mice. Animals were sacrifice 28 days post injection (dpi) and DG and OB structure were analyzed (**B**) Quantification of (**A**) as a percentage of double-labeled GFP-Cre/RFP$^+$ normalized to the total number of RFP cells (n=3). Student T-test was performed relative to control. Error bars represent SEM.

respectively. However, these studies suggest an increase in apoptosis in postnatal VZ/SVZ, in the compound mutants, which could be indicative of VZ/SVZ disruption.

6.7. Atypical E2fs are required for proper postnatal differentiation of ependymal cells

Ventriculomegaly is associated with disruption of the VZ/SVZ. Loss of the VZ during embryonic neurogenesis results in depletion of NSCs, increased cell death and enlargement of the LV. However, postnatal disruption of the VZ/SVZ is associated with ependymal denudation, also know as disruption or loss of ependymal cells (Jimenez, Tome et al. 2001, Wagner, Batiz et al. 2003, Chae, Kim et al. 2004, Rodriguez, Guerra et al. 2012). Since we detect congenital ventriculomegaly associated with cell death, we questioned whether the ependymal cells were altered in the compound mutants. In certain human cases of ventriculomegaly, ependymal cell denudation precedes lateral ventricle dilation and could possibly trigger the onset of ventriculomegaly (Dominguez-Pinos, Paez et al. 2005, Sival, Guerra et al. 2011), thus, we suspected that ventriculomegaly in the cDKO mice may be caused by defects in ependymal cells. We performed immunohistochemistry on whole-mount preparations of the LV surface to visualize ependymal cells. The SVZ of cDKO and control mice were dissected and stained for S100-beta, a marker of the ependymal cell layer in the VZ neural stem cell niche (Figure 1.3), at P5, P8, P21 and 3 months (adult) (Figure 6.8A). In the controls, we found a progressive increase in ependymal cells in control brains from P5 to P21. However, no change in the number of S100-beta^{+} ependymal cells was observed between P8 and P21 in the compound mutants (Figure 6.8B). In the mutants, we detected

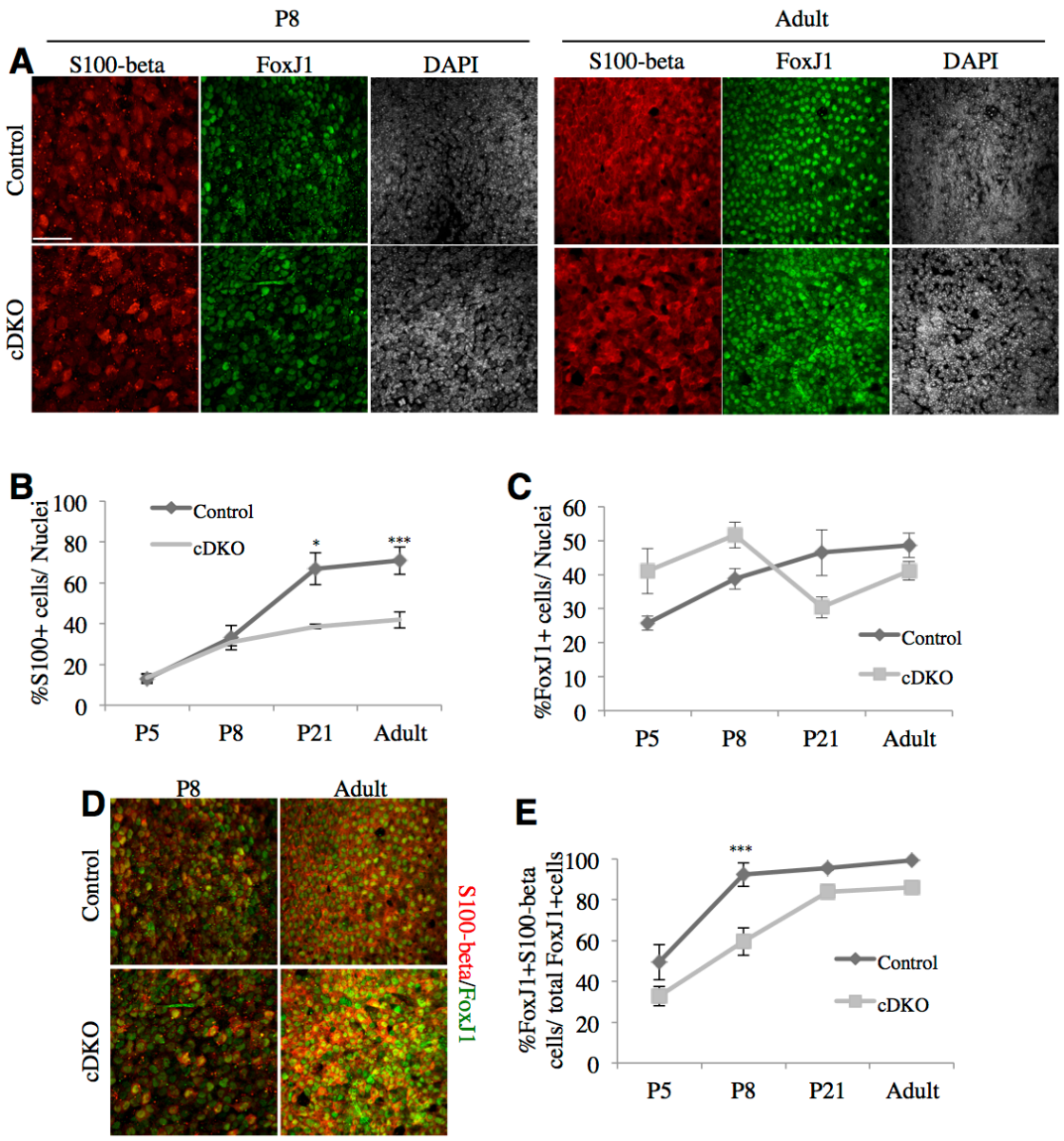


Figure 6.8: Ependymal cell differentiation abnormal in cDKO animals.

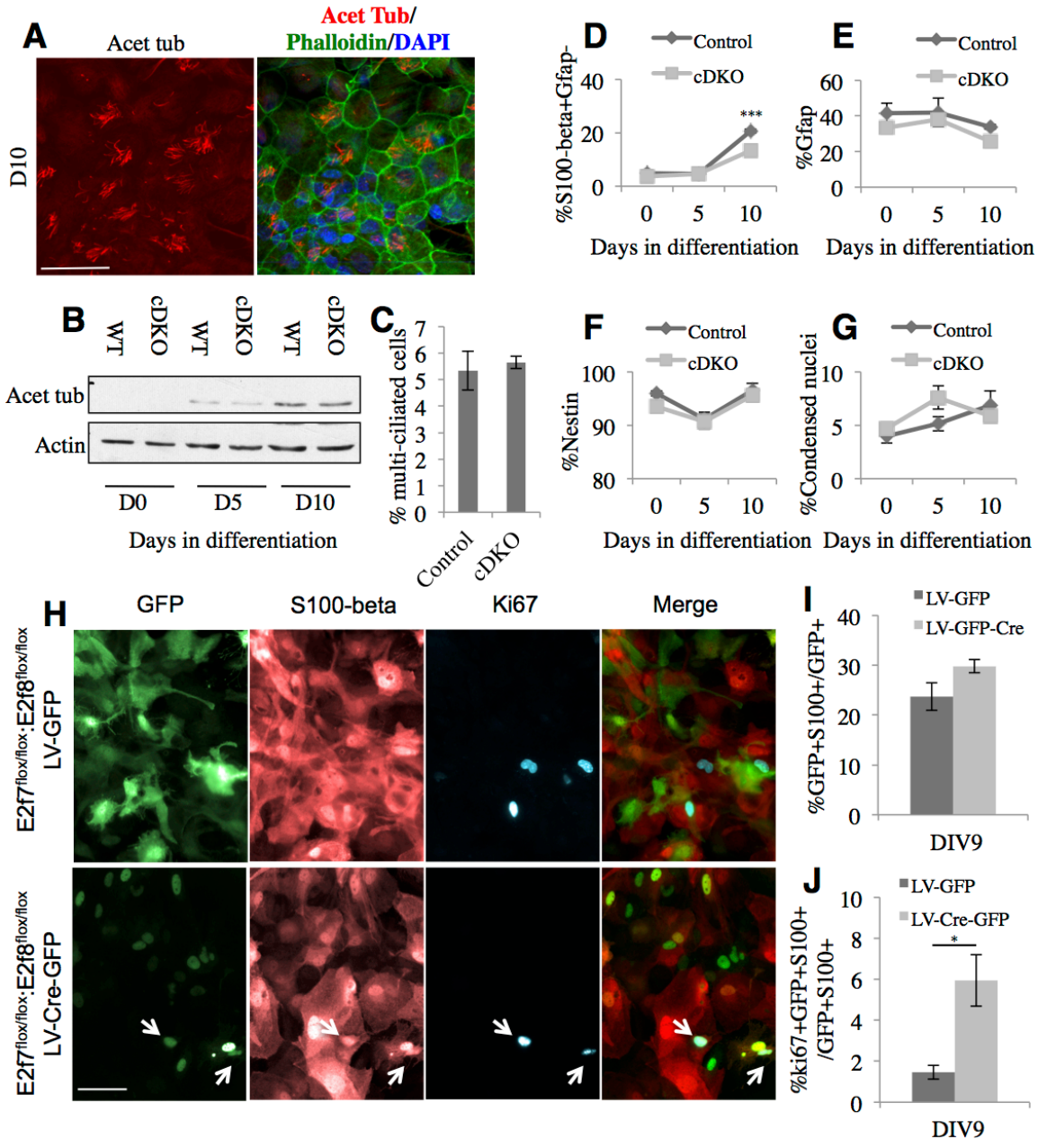
(**A**) Whole-mount preparation of the wall of the lateral ventricle of control and cDKO of P8, 3 month-old (adult) mice was stained for S100-beta, FoxJ1 and DAPI. (**B**) Quantification of the number of S100-beta$^+$ and (**C**) FoxJ1 within defined area in the SVZ of P5, P8, P21 and adult. Results are expressed as a percentage of DAPI nuclear staining. (**D**) Overlay of wholemount immunostaining for S100-beta (red) and FoxJ1 (green) described in (**A**). (**E**) Quantification of the number of cells expressing both S100-beta and FoxJ1 within a defined area in the SVZ. Results are expressed as a percentage of FoxJ1$^+$ cells. P5 (Control, n= 3; cDKO, n=4), P8 (Control, n=3; cDKO, n=3), P21 (control n=3,; cDKO, n=5) and adult mice (Control, n=9; cDKO, n=9). Two-way ANOVA with Bonferroni was performed for multiple comparisons. Scale bar 50μm. Error bars represent SEM. $^*p<0.05$, $^{***}p<0.001$.

a 40% decrease in S100-beta expressing cells compared to the control brains at 3 weeks of age (Figure 6.8B), suggesting that loss of atypical E2fs results in denudation of the ependymal cells.

Animal models with defects in ependymal cell differentiation have also been shown to exhibit ependymal cell denudation and ventriculomegaly (Jacquet, Salinas-Mondragon et al. 2009, Mirzadeh, Han et al. 2010, Tissir, Qu et al. 2010, Lavado and Oliver 2011). In rodents, ependymal cell differentiation commences at birth (Spassky, Merkle et al. 2005), which coincides with the onset of congenital ventriculomegaly in the *E2f7/E2f8* compound mutant mice. Because, ependymal cells complete their maturation by 21-days postnatal (Spassky, Merkle et al. 2005), we then sought to determine whether ependymal cell differentiation was altered in the cDKO mice. During ependymal cell maturation, ependymal cells differentiate from monociliated to multiciliated cells (Delgehyr, Meunier et al. 2015). FoxJ1 expression is restricted to cells possessing multicilia (Hackett, Brody et al. 1995, Lim, Zhou et al. 1997, Blatt, Yan et al. 1999, Ostrowski, Hutchins et al. 2003). Although immunostained whole-mount SVZ preparations stained for FoxJ1 showed no significant changes, an abnormal expression pattern was noted in the compound mutants (Figure 6.8A&C). As ependymal differentiation progresses through time, the majority of mature adult S100-beta$^+$ ependymal cells will also express FoxJ1 (Jacquet, Salinas-Mondragon et al. 2009). Interestingly, at P8, we detect a 35% decrease in the percentage of S100-beta$^+$/FoxJ1$^+$ double-labeled cells within the FoxJ1 population of the *E2f7/E2f8* mutants (Figure 6.8D-E), suggesting that ependymal dell differentiation may be delayed in the *E2f7/E2f8* mutants.

Considering that we detected denudation in S100-beta-expressing ependymal cells *in vivo*, we investigated whether ependymal cells could differentiate properly in the absence of atypical E2fs. To address this question, we differentiated ependymal cells *in vitro* (Spassky, Merkle et al. 2005, Delgehyr, Meunier et al. 2015). To assess the efficiency of the culture system, we first isolated radial glial cells (RGCs) from the SVZ of P3 WT mice and conducted immunocytochemical analysis at 10 days *in vitro* (D10). By D10, we could distinguish clusters of multiciliated cells in WT cultures, marked by acetylated tubulin (Acet tub). Phalloidin staining, which labels filamentous actin (F-actin), delineates the cell border for better visualization of ependymal cells (Figure 6.9A). Once this *in vitro* model system was established, we assessed the ability of RGCs isolated from *E2f7*/*E2f8* compound mutants to differentiate into ependymal cells *in vitro*. RGCs from both the control and cDKO were assessed after 10 days *in vitro* by immunocytochemistry and western blotting with an antibody to detect acetylated tubulin. Protein levels revealed a progressive increase at D0, D5 and D10 of acetylated tubulin in both the control and cDKO cultures, which coincides with ependymal cell differentiation (Figure 6.9B) and a similar percentage of multiciliated cells at D10 in both cDKO and the control cultures (Figure 6.9C). These results suggest that *E2f7*/*E2f8* mutant cultures can form multi-ciliated cells *in vitro*.

Given that loss of atypical E2fs leads to denudation of S100-beta^{+} ependymal cell population *in vivo*, we asked whether denudation could be caused by delayed ependymal cell differentiation or reduction in S100-beta^{+} ependymal cell population. It is important


A
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D10
Days in differentiation
C
% multi-ciliated cells
Control
cDKO
D
Control
cDKO
%S100-beta+Gfap-
***
Days in differentiation
E
Control
cDKO
%Gfap
Days in differentiation
F
Control
cDKO
%Nestin
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G
Control
cDKO
%Condensed nuclei
Days in differentiation
H
GFP
S100-beta
Ki67
Merge
E2f7flox/flox:E2f8flox/flox
LV-GFP
E2f7flox/flox:E2f8flox/flox
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I
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LV-GFP-Cre
%GFP+S100+/GFP+
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Figure 6.9

Figure 6.9: Ependymal cell defects observed in primary *in vitro* cDKO ependymal cell cultures.

(**A**) Immunostaining of cultured ependymal cells at 10-days (D10) *in vitro*, with antibodies marking multiciliated cells, acetylated tubulin (Acet Tub), cytoskeleton, phalloidin and nuclei (DAPI). (**B**) Western blots of acetylated tubulin shows increase in both control (n=3) and cDKO (n=3) during ependymal cell differentiation. (**C**) Quantification of the percentage of multiciliated cells at D10 from ependymal cell differentiation culture. Results represented as total DAPI proportion (n=3) for both genotypes. (**D-G**) Quantification of immunostained cultures at 0, 5 and 10-days post differentiation with the indicated cell-type markers (n=3) for both control and cDKO. $E2f7^{flox/flox};E2f8^{flox/flox}$ radial glial cultures were infected at time of differentiation with a CMV-GFP or CMV-Cre-GFP lenti-virus. Cells were maintained in serum-free media to induce differentiation to ependymal cells. (**H**) Immunostaining of the resulting cultures for GFP, S100-beta and ki67 at 9-days post differentiation. (**I**) Quantification of (**H**) as a percentage of double-labeled $GFP^+/S100\text{-}beta^+$ cells per total GFP infected cells and (**J**) of triple labeled $Ki67^+/GFP^+/S100\text{-}beta^+$ cells per total S100-beta infected GFP cells. (**C&I-J**) Student T-test was performed relative to control and (**D-G**) Two-way ANOVA with Bonferroni was performed for multiple comparisons. Scale bars 50 μm. Error bars represent SEM. $^{*}p<0.05$, $^{***}p<0.001$.

to note, S100-beta$^+$ is also expressed in mature astrocytes (Yasuda, Tateishi et al. 2004). In the postnatal SVZ, S100-beta is absent in differentiating and mature astrocytes, however S100-beta is re-expressed in mature cortical astrocytes (Dyck, Van Eldik et al. 1993, Vives, Alonso et al. 2003, Yasuda, Tateishi et al. 2004, Steiner, Bernstein et al. 2007). *In vivo*, ependymal cells express S100-beta and nestin and are negative for GFAP (Figure 1.3), we therefore quantified ependymal-like cells *in vitro* as a proportion of S100-beta$^+$/GFAP$^-$ cells within the nestin$^+$ population to minimize glia contamination within our counts. In the cDKO cultures, we detected a 35% decrease in ependymal-like cells by 10 days *in vitro* (Figure 6.9D), without discerning differences in the GFAP or nestin expressing cells (Figure 6.9E-F). To determine if this decrease was due to apoptosis of the ependymal cells, we examined the level of cell death by quantifying the percentage of condensed nuclei within the cultures and found no differences between genotypes (Figure 6.9G). These data demonstrate that although cDKO cultures form multiciliated cells, we detect a decrease in ependymal-like cells *in vitro*. This suggests that the effect of atypical E2fs on the proportion of ependymal-like cells in culture maybe be due to a cell autonomous defect.

To examine whether loss of atypical E2fs can affect S100-beta$^+$ ependymal-like cells in culture in a cell autonomous fashion, we infected cultured RGCs from the SVZ of P3 *E2f7$^{flox/flox}$;E2f8$^{flox/flox}$* mice with either i) lentivirus expressing Cre recombinase and GFP to delete atypical E2fs or ii) with a control vector expressing-GFP and assessed 9 days *in vitro*. We quantified the percentage of S100-beta$^+$ infected (GFP) cells in cultures and detected no changes between LV-GFP and LV-Cre-GFP cultures (Figure 6.9I), suggesting that decrease in S100-beta$^+$ ependymal-like cells may be due to a non-cell

autonomous effect. In cases of ventriculomegaly, a delay in ependymal cell differentiation results in ectopic proliferation of normally post-mitotic ependymal cells (Lavado and Oliver 2011). To examine whether loss of atypical E2fs could lead to ectopic expression of proliferation of ependymal-like cells in culture, we then stained for S100-beta, GFP and Ki67. We observed a 4-fold increase in $Ki67^{+}$/$S100\text{-}beta^{+}$ in *E2f7*/*E2f8*-deficient ependymal cell cultures, which were rarely visible in control cultures (Figure 9H&J). These results, in combination with the *in vivo* results suggest that ependymal cells in cDKO animals, while being able to differentiate into multiciliated cells, may have a delay in differentiation.

6.8. Loss of atypical E2fs leads to ectopic proliferation of ependymal cells *in vivo*

Since we detected Ki67 expression in S100-beta-expressing cells following acute knockout of atypical E2fs *in vitro*, suggesting delayed ependymal cell differentiation, we questioned whether long-term loss of atypical E2fs leads to a proliferation defect. To determine if S100-beta-labelled cells were ectopically proliferating *in vivo,* we immunostained P8 and adult control and cDKO mice for S100-beta and Ki67. We observed with Ki67, a prominent 20-fold and 5-fold increase in the number of double-labelled $Ki67^{+}$/$S100\text{-}beta^{+}$ cells in the P8 and adult *E2f7*/*E2f8* mutant mice, respectively (Figure 6.10A-B). However, minor population of retinal cells retain Ki67-expression in differentiated neurons after the final mitosis (Pacal and Bremner 2012, Pacal and Bremner 2014). To determine the proliferative nature of post-mitotic ependymal cells, 2 month old (adult) mice were injected with a single dose of BrdU, a time which corresponds to non-dividing ependymal cells. To visualise proliferation within the

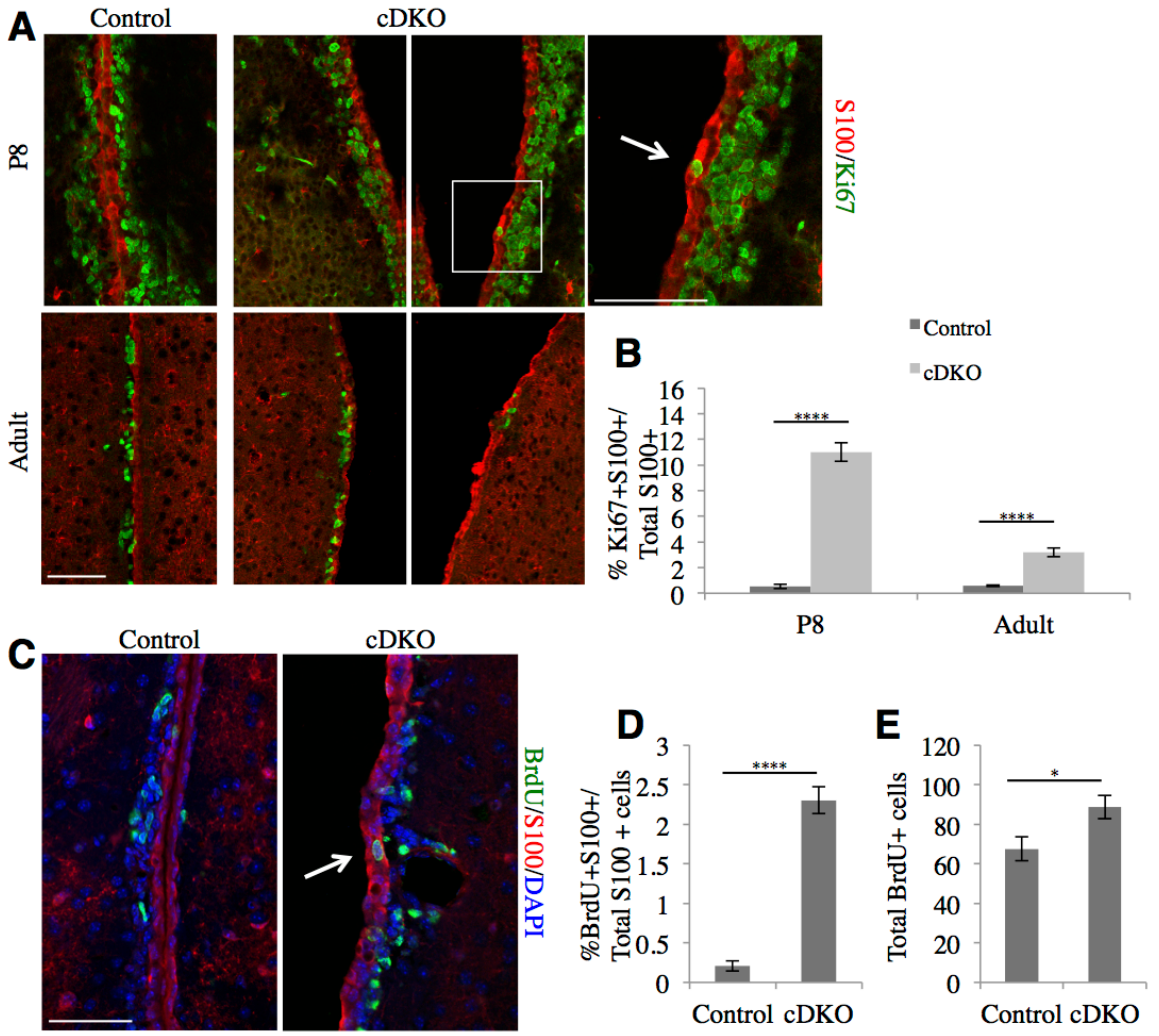

Figure 6.10: Deletion of atypical E2fs results in ectopic ependymal cells proliferation.

(**A**) Immunostaining of Ki67 and S100-beta shows increase of Ki67^{+} cells co-labeled with ependymal cell marker S100-beta in the cDKO mice. (**B**) Quantification of (**A**), represented as the percentage of Ki67^{+}/S100-beta^{+} co-labelled over total S100-beta population around the LV, P8 (n=4 control, n=3 cDKO) and 7-week and old animals (n=7 control, n=8 cDKO). (**C-E**) To assess proliferation 3 month and older animals were injected with BrdU and sacrificed 2-h later. Ependymal cells were stained for S100-beta and double labeled BrdU^{+}/S100-beta^{+} cells were counted and quantified (n= 7 control, n=8 cDKO). Student T-test was performed relative to control. Scale bars 50 μm. Error bars represent SEM. $^{*}p<0.05$, $^{****}p<0.0001$.

ependymal cell layer, animals were sacrificed 2h later and cells undergoing S-phase during the time of injection were double-labelled with BrdU and S100-beta (Figure 6.10C). In the VZ/SVZ, only ependymal cells express S100-beta (Yasuda, Tateishi et al. 2004, Spassky, Merkle et al. 2005, Steiner, Bernstein et al. 2007). Quantification of the double-labelled cells revealed an 11-fold increase in the percentage of BrdU and S100-beta expressing cells in the VZ of the cDKO mice (Figure 6.10C-D). We also detected a slight but significant 1.3-fold increase in the total number of $BrdU^+$ cells in the VZ-SVZ of the compound mutant (Figure 6.10E). In summary, consistent with the *in vitro* ependymal cell culture results, loss of atypical E2fs leads to ectopic proliferation characteristic of ependymal cells. Based on these results, we question whether atypical E2fs may have a specific role in ependymal cell regulation.

6.9. Increase in astrocytes within ependymal cell population in the cDKO mice

Ependymal that exhibit delayed cell differentiation have been shown to undergo ectopic proliferation (Lavado and Oliver 2011). However, post-mitotic ependymal cells have also shown ectopic proliferation in response to stress or injury, such as following a stroke (Zhang, Zhang et al. 2007, Carlen, Meletis et al. 2009). Stress or injury, ependymal cell denudation and delay in ependymal cell differentiation have all been associated with the appearance of reactive astrocytes, also known as gliosis (Bruni, Del Bigio et al. 1985, Sarnat 1995, Lavado and Oliver 2011, Shook, Lennington et al. 2014). Gliosis is manifested as an increase in the number of astrocytes in inappropriate locations, including the ependymal cell layer. Since GFAP-expressing astrocytes do not normally associate with the S100-beta cells in the SVZ (Yasuda, Tateishi et al. 2004,

Steiner, Bernstein et al. 2007), we investigated whether gliosis was present in the ependymal cell barrier in the compound mutants. While control mice showed minimal expression of astrocyte marker GFAP within the S100-beta-expressing ependymal cells (Figure 6.11A), we detected a 3-fold, 7-fold and 14-fold increase in the percentage of GFAP-expressing cells within the S100-beta–expressing cells of the P3, P8 and adult cDKO mice, respectively (Figure 6.11A-B). Gliosis is seen as a natural brain response to injury (Little and O'Callagha 2001) and a recent study showed that ventricular surface gliosis is directly associated with ventricular expansion (Shook, Lennington et al. 2014). The presence of gliosis within the ependymal cell barrier around the time of onset of ventricular dilation in cDKO mice is indicative of ependymal cell dysfunction underlying ventriculomegaly.

Under normal conditions, the VZ is composed of a single layer of post-mitotic ependymal cells (Spassky, Merkle et al. 2005). However, they can acquire radial-glial characteristic in response to compromised ependymal cell barrier (Kuo, Mirzadeh et al. 2006, Zhang, Zhang et al. 2007, Luo, Shook et al. 2008, Lavado and Oliver 2011, Capilla-Gonzalez, Cebrian-Silla et al. 2014). To assess whether the ependymal cell barrier is compromised in the *E2f7*/*E2f8* mutants, we performed immunostaining on P8 and adult section for nestin (expressed in ependymal and RGCs) and GFAP (expressed in RGCs and cortical astrocytes) (Figure 1.3). We found a 2-fold and 5-fold increase in the percentage of GFAP expression within the nestin-expressing cells lining the wall of the lateral ventricle in the P8 and adult *E2f7*/*E2f8* mutants (Figure 6.11C-D). Furthermore, analysis of double-labeled GFAP- and nestin-expressing cells at the surface of the lateral

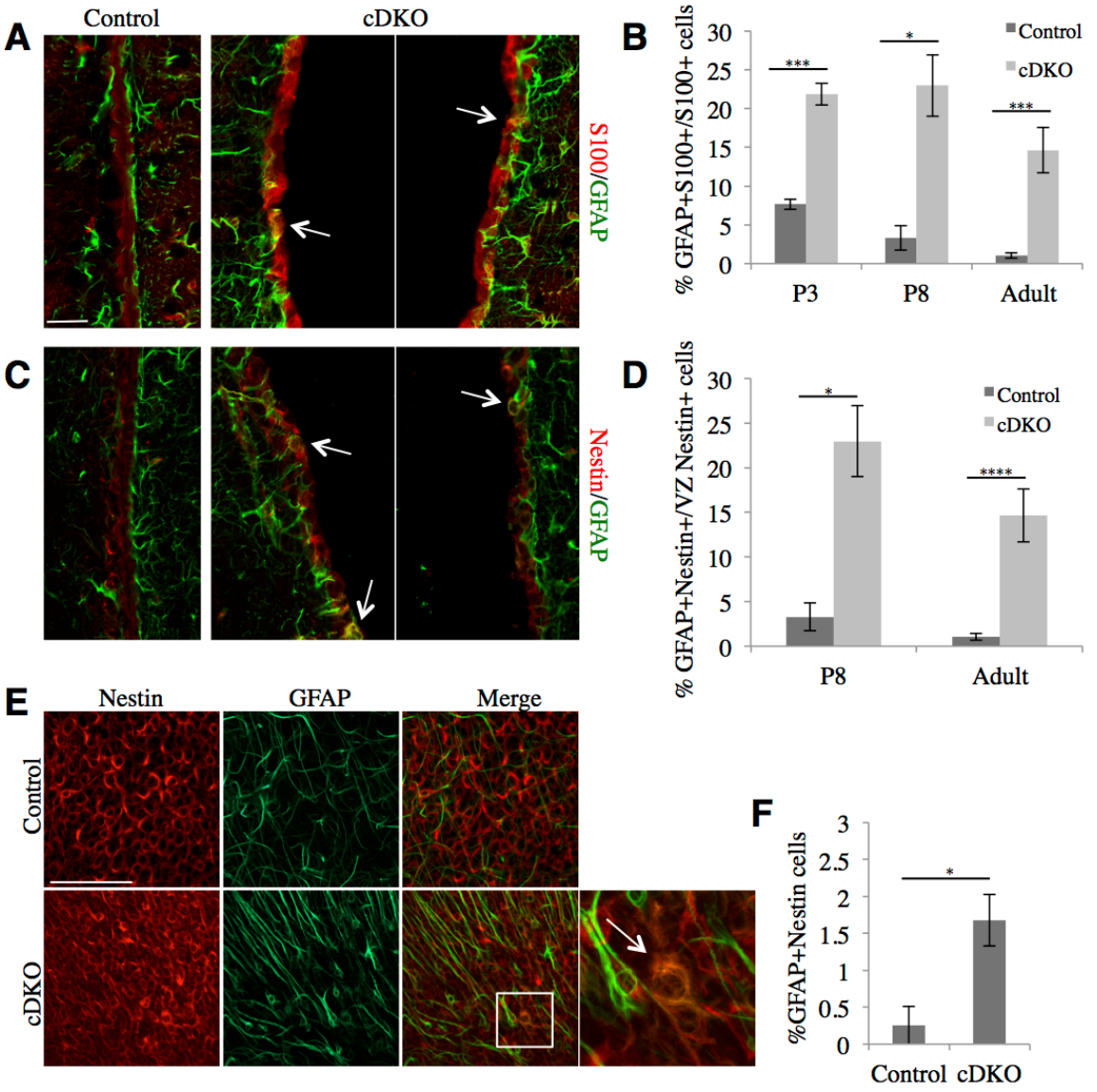

Figure 6.11: Loss of atypical E2fs leads to gliosis of the ependymal cell barrier.

(A&C) Coronal sections of control and cDKO animals of 7-week and older mice were immunostained for both GFAP and S100-beta (**A**) or GFAP and nestin **(C)**. **(B)** The percentage of GFAP/S100-beta$^+$ population was quantified within the total S100-beta$^+$ cells and (**D**) GFAP+ of the nestin$^+$ population was quantified within the cell lining the wall of lateral ventricle (n= 7 control, n=8 cDKO). Student T-test was performed relative to control. Scale bar 25 μm. Error bars represent SEM. ***p<0.001, ****p<0.0001 **(E)** Whole-mount preparation of the wall of the LV was immunostained with nestin and GFAP in control (n=3) and cDKO (n=3) of 3-month-old adult animals. **(F)** The percentage of double-labeled GFAP$^+$/nestin$^+$ cells within the DAPI population was increased in the cDKO compared to control. Student T-test was performed relative to control. Scale bar 50 μm. Error bars represent SEM. *p<0.05, ****p<0.0001.

ventricle by whole-mount immunohistochemistry also showed a 6.6-fold increase in the 3 month old cDKO mice (Figure 6.11E-F). These results are indicative of the presence of RGCs within the ependymal cell barrier. The ependymal cell barrier is now composed of a mixed population of ependymal cells, RGCs and astrocytes. Altogether, these results show absence of atypical E2fs results in the compromised ependymal cell barrier and gliosis at the ventricular surface, this may be the underlying cause of ventriculomegaly in the *E2f7/E2f8* mutants.

6.10. Deletion of atypical E2fs results in abnormal motile cilia

Motile cilia, a hallmark of ependymal cells, extend into the ventricular cavity and facilitate intraventricular CSF flow (Nelson and Wright 1974, Bruni, Del Bigio et al. 1985, Eley, Yates et al. 2005, Bisgrove and Yost 2006, Jimenez, Dominguez-Pinos et al. 2014, Delgehyr, Meunier et al. 2015). In addition to gliosis and a compromised ependymal cell barrier, impairment in motile cilia function has been associated with ventriculomegaly (Eley, Yates et al. 2005, Bisgrove and Yost 2006, Tissir, Qu et al. 2010, Sival, Guerra et al. 2011, Roales-Bujan, Paez et al. 2012, Jimenez, Dominguez-Pinos et al. 2014, Ohata, Nakatani et al. 2014). The complete loss of ependymal motile cilia has been shown to cause ventriculomegaly (Jacquet, Salinas-Mondragon et al. 2009). We therefore questioned whether loss of atypical E2fs was compatible with *in vivo* formation of motile cilia in ependymal cells. Similar to S100-beta, the intermediate filament protein, vimentin, is highly expressed in multiciliated ependymal cells (Figure 1.3) (Kirik and Korzhevskii 2013). By immunostaining whole-mount ventricular preparation and coronal sections for acetylated tubulin and vimentin to label motile cilia

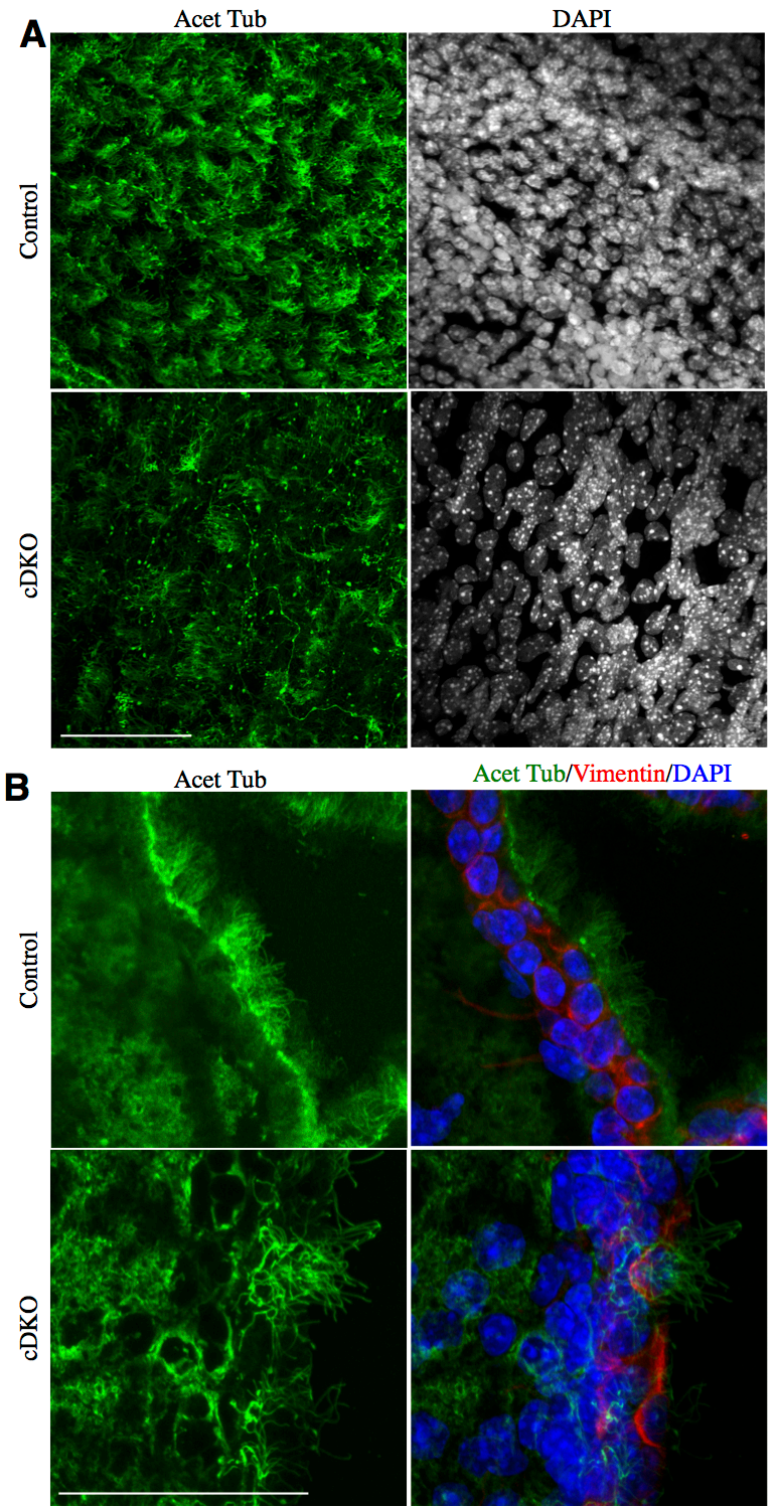

Figure 6.12: Mutant cDKO mice show aberrant motile cilia

(**A**) Whole-mount immunostaining of control and cDKO adult mouse were stained for cilia marker, acetylated tubulin. (**B**) Coronal sections of control and cDKO adult mice were stained for acetylated tubulin, ependymal cell marker, vimentin, and nuclear stain represented by DAPI. Scale bars 50μm.

and ependymal cells, we confirmed that, although adult compound mutants have the capacity to generate multiciliated ependymal cells (Figure 6.12A-B), loss of atypical E2fs leads to disorganization of the motile cilia. In the control brains, we observed motile cilia organised in clusters on the apical ependymal cell surface and they protruded into the cerebral ventricle in a polarized fashion. Loss of atypical E2fs alters acetylated tubulin organization, where aberrant orientation of the motile cilia is observed. Furthermore, motile cilia are ectopically present within the brain tissue rather than extended into the lateral ventricles of the cDKO mice (Figure 6.12B). Although, we demonstrated that *E2f7/E2f8* mutants have the ability to generate multiciliated ependymal cells, these results revealed abnormal morphological defects in motile cilia orientation and organization.

In summary, these results reveal a novel function for atypical E2fs in maintenance of the ependymal cell barrier. Loss of atypical E2fs leads to congenital ventriculomeglay and may be key regulators for the development of the ependymal cell barrier. The underlying cause of congenital ventriculomegaly in the compound mutants is still unknown. However, it is becoming increasingly evident that the molecular mechanism important for the formation of the ependymal cell barrier may provide insight into the development of this phenotype.

6.11. Neither *E2f1* nor *E2f3* rescues ventriculomegaly in the cDKO mice

We sought to identify the molecular mechanism contributing to congenital ventriculomegaly in the cDKO mice. Previous work has shown that atypical E2fs counteract or balance the activity of the canonical activator E2fs, E2F1-3 (Li, Ran et al. 2008, Chen, Ouseph et al. 2012, Ouseph, Li et al. 2012). The tightly coordinated feed-

back loop regulating the canonical activator and atypical E2fs is critical in maintaining proper cell cycle progression. Increased apoptosis resulting from loss of atypical E2fs, can be restored to control levels by concomitant deletion of *E2f1* or *E2f3* (Li, Ran et al. 2008, Ouseph, Li et al. 2012). In order to identify whether increased activity of the activator E2fs in the absence of atypical E2fs could contribute to the underlying cause of ventriculomegaly, we generated cTKO mice deficient for either *E2f1* or *E2f3* (Table 5.1&6.1B). We examined the brains of 3-month old animals of the following genotypes using cresyl violet staining for the presence of enlarged lateral ventricles: control, cDKO, 7:8:1:cTKO (cDKO;$E2f1^{-/-}$) and 7:8:3:cTKO (cDKO;$E2f3^{flox/flox}$) (Table 5.1). Similar to cDKO, we found that the LV were enlarged in the 7:8:1:cTKO and 7:8:3:cTKO mouse brains compared to the control (Figure 6.13A). Measurement of the ventricular area revealed a 8.8-fold, 9-fold and 10-fold increase in cDKO, 7:8:1:cTKO and 7:8:3:cTKO brains, respectively (Figure 6.13B), suggesting that removal of *E2f1* or *E2f3* are not sufficient to rescue congenital ventriculomegaly in the *E2f7/E2f8* mutants. Activating E2fs share similar structure and functions and thus, *E2f1* can compensate for *E2f3* loss (Tsai, Opavsky et al. 2008). E2f1 induces retinal neuronal cell death in rods, bipolar and ganglion cells while; *E2f2* drives cone apoptosis in the *Rb1/p107* mutants (Chen, Opavsky et al. 2007, Chen, Chen et al. 2013). We cannot rule out the possibility of E2f2 involvement or redundant action of E2f1, E2f2 and/or E2f3 in ependymal cell death.

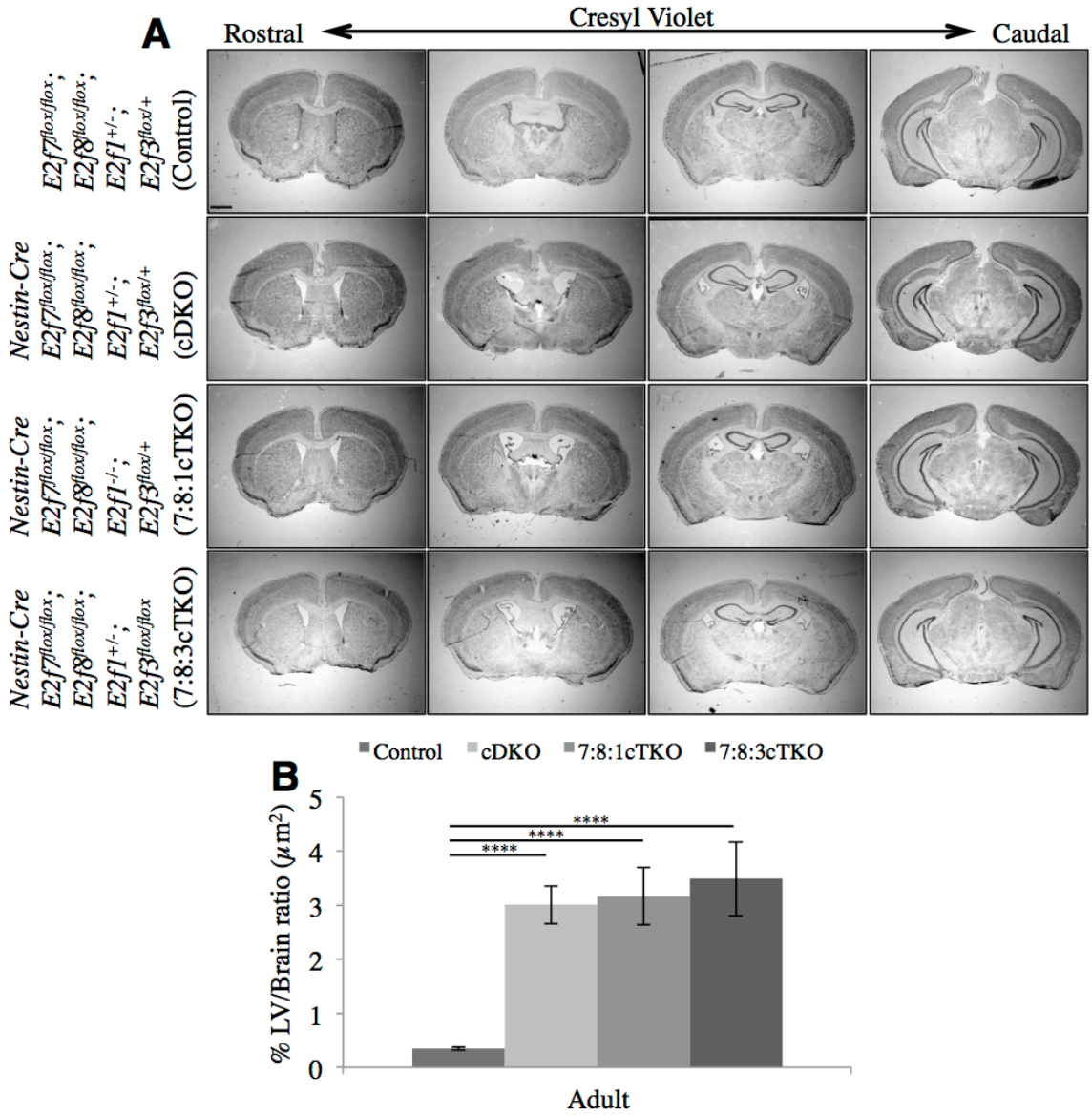

Figure 6.13: Loss of *E2f1* or *E2f3* does not rescue enlargement of the lateral ventricle in cDKO animals

(A) Cresyl violet staining of brain coronal sections from 8 weeks adult mice of the indicated genotype. **(B)** The area of both right and left LV was measured and divided by the total area of the brain (%LV/Brain ratio) of the indicated genotypes (n=12 control, n=10 cDKO, n=3 7:8:1cTKO, n=3 7:8:3TKO). One-way ANOVA with Tukey's was performed to compare the %LV/Brain ratio. Scale bar 1 mm. Error bars represent SEM. $^{****}p<0.0001$.

6.12. Loss of atypical E2fs leads to increased ependymal cell adherens junction molecules

In an attempt to uncover the molecular events connecting loss of atypical E2fs to ventriculomegaly, we investigated possible molecules involved in VZ denudation. Adherens junctions between ependymal cells have been shown to be required for maintenance of the ependymal cell barrier since mice with impairment in adherens molecules develop ventriculomegaly associated with comprised ependymal cell barrier and gliosis at the ventricular surface (Chenn, Zhang et al. 1998, Chae, Kim et al. 2004). Cadherins are integral components of adherens junctions which enable cell adhesion by forming an intercellular bridge, and defect in cadherins results in VZ/ependymal denudation and ventricuomegaly (Lippoldt, Jansson et al. 2000, Vaezi, Bauer et al. 2002, Rodriguez, Guerra et al. 2012, Guerra, Henzi et al. 2015). Given that our data revealed substantial ependymal cell barrier abnormalities, we questioned whether adherens junction molecules were misexpressed in the *E2f7/E2f8* mutants. To address this question, we performed immunohistochemistry on P3 sections, at the time that corresponds to ventriculomegaly and stained for adherens molecule markers, N- and E-cadherin (Figure 6.14A-B). Interestingly, we observe increased expression of both N- and E-cadherin at the apical surface of the lateral ventricle of the compound mutants. Selective deregulation of cadherins expression at the ventricular surface leads us to question whether accumulation of cadherins could provoke ependymal denudation.

Previous work has shown that Numb and NumbL is essential for the maintenance of cadherin-based adhesion of the ependymal cell barrier (Kuo, Mirzadeh et al. 2006, Rasin, Gazula et al. 2007). During postnatal establishment of the SVZ neural stem cell

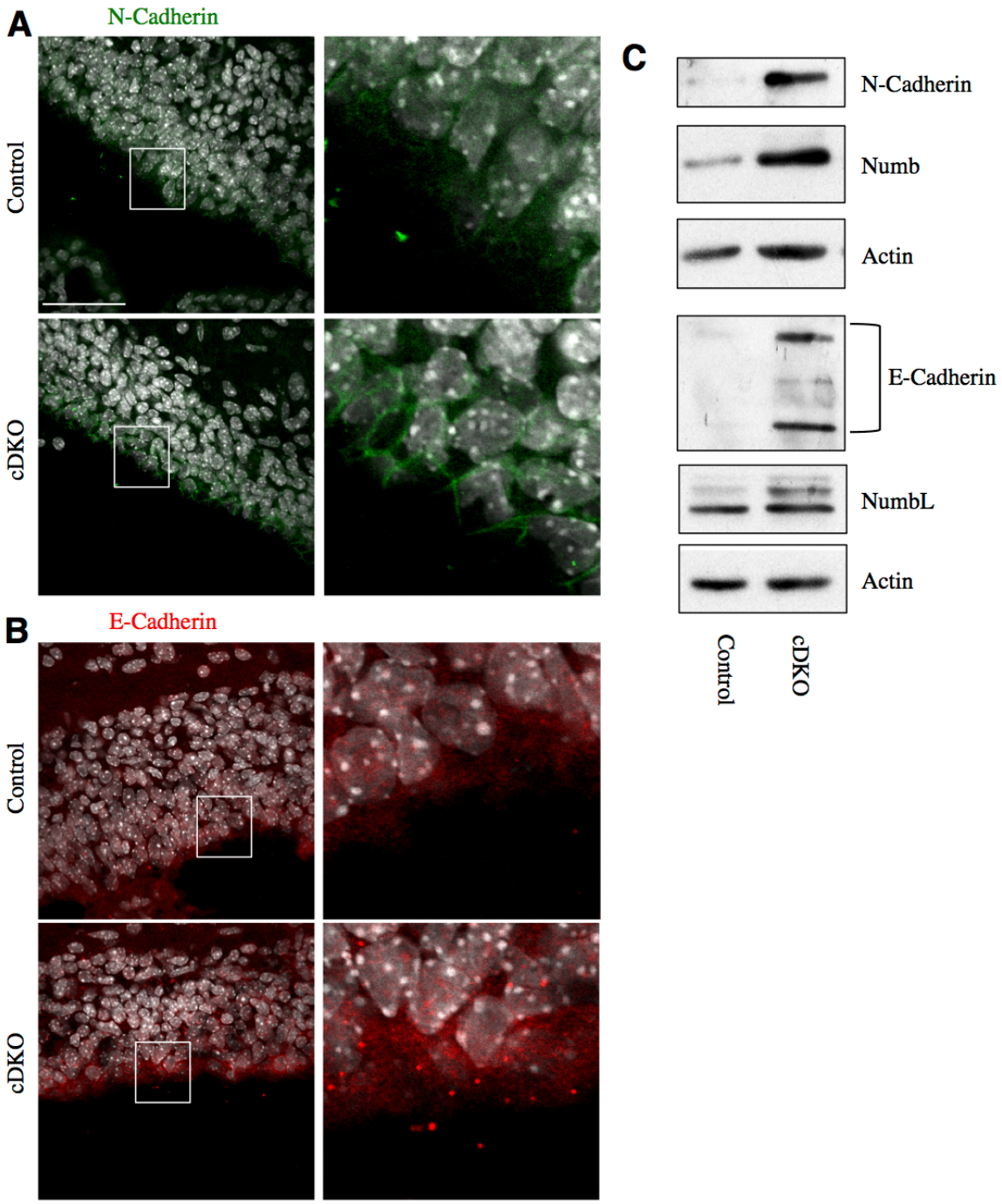

Figure 6.14: Loss of atypical E2fs results in increase level of Numb and cadherins

Immunostaining of N-cadherin (**A**) and E-cadherin (**B**) in P3 cDKO cortical lateral ventricle (**C**) Western blots of N-cadherin, E-cadherin (precursor: 135 kDA and mature form 120/80 kDa), Numb and NumbL in P3 SVZ tissue normalized to actin, (n=3) for both genotypes. Scale bar 50 μm.

niche, Numb is required for the proper localization of cadherins to the ependymal cell barrier (Rasin, Gazula et al. 2007). Postnatal deletion of *Numb* and *NumbL* results in impairment of cadherin-mediated adhesion (Kuo, Mirzadeh et al. 2006, Rasin, Gazula et al. 2007). Interestingly, the cDKO mutant phenotype is very similar to the *Numb/NumbL* mutant mice, which also exhibit ventricular gliosis and congenital ventriculomegaly. Thus we asked whether Numb level was altered in the DKO mutants. We conducted Western blotting in P3 SVZ dissected tissue. Our results revealed increased expression of N- and E-cadherins as well as Numb and NumbL protein levels in the *E2f7/E2f8* mutants (Figure 6.14C). These results suggest that upregulation of Numb/NumbL in the compound mutants could trigger accumulation of cadherins at the apical surface of the lateral ventricle, thus disrupting the development of the ependymal cell barrier.

6.13. E2f4 regulates *Gli3*

In order to define the molecular mechanism by which atypical E2fs regulate development of the ependymal cell barrier, we investigated known signalling pathways involved in Numb mediated formation of adherens junctions. *Gli3*, a negative regulator of the Shh pathway, regulates E-cadherin localization to the ventricular surface by preventing LNX2-dependent Numb/NumbL degradation (Wang, Kane et al. 2014). We have previously shown a genetic interaction between *E2f4* and *Gli3* and the Shh pathway, and have revealed that E2f4 is required for ventral telencephalon development through modulation of the Shh pathway (Ruzhynsky, McClellan et al. 2007), although direct regulation of *Gli3* by E2f4 has never been shown. Based on these findings, we hypothesized that E2f4 regulates the *Gli3* expression in the developing telencephalon.

E2f4 together with p107 and atypical E2fs modulate similar subsets of neuronal differentiation genes (Ghanem, Andrusiak et al. 2012), suggesting the possibility that atypical E2fs could regulate *Gli3* similar to the repressor complex E2f4. To investigate this, we conducted a ChIP on chip bioinformatics analysis to determine promoters bound by E2f4 (Julian, Liu et al. 2015). We found enrichment of E2f4 binding to the 5´ flanking region of the *Gli3* gene (Figure 6.15A). By searching for the previously defined E2f consensus site (BKTSSCGS) (Rabinovich, Jin et al. 2008) within the publically available DNA sequence Mulan/rVista software, the 5´ region of mouse *Gli3* gene was examined for E2f binding sites. One broad-spectrum BKTSSCGS E2f motif could be found in the *Gli3* promoter region, corresponding to the ChIP-on-chip enrichment region (Figure 6.15A). Closer examination revealed a broad range of non-classical E2fs binding sites located within 1.9-kb of the promoter region (Figure 6.15D). To confirm the relevance of these binding sites, we tested whether E2f4 could bind to the *Gli3* promoter using a conventional chromatin immunoprecipitation (ChIP) assay. Chromatin was isolated from WT neurosphere culture and then immunoprecipitated using an antibody directed against E2f4, followed by PCR amplification with primers designed to encompass the defined BKTSSCGS E2f consensus site on the *Gli3* gene. The PCR shows E2f4 binding to the *Gli3* promoter (Figure 6.15-A-B). Given that E2f4 classically functions as a transcriptional repressor, we then asked whether *Gli3* mRNA was upregulated in the *E2f4*$^{-/-}$ mutants. We performed qRT-PCR on E12.5 tissue extracted

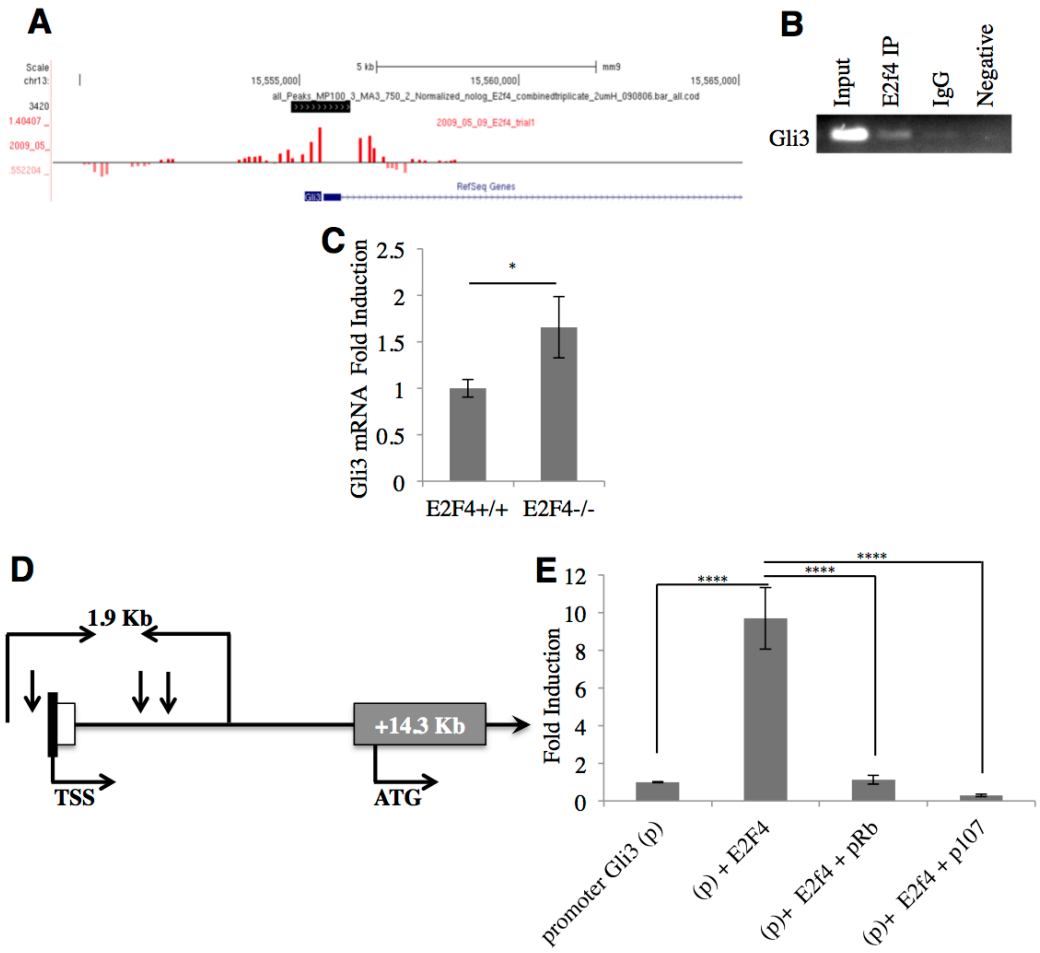

Figure 6.15: E2f4 regulates *Gli3* expression

(**A**) Binding-peak profile for E2f4 ChIP-on-chip experiments, generated with the UCSC Genome Browser (http://genome.ucsc.edu/). E2f4 binding peaks extend throughout the promoter region of the *Gli3* gene. (**B**) ChIP was performed on neurospheres isolated from ganglionic eminence of E14.5 wild-type embryos. Immunoprecipitation was performed using an antibody specific to E2f4, followed by PCR amplification of the *Gli3* gene (representative gel of three independent experiments). (**C**) Quantitative real-time PCR analysis of *Gli3* showed increased expression in *E2f4* mutant compared to WT at E12.5 in tissue collected from the telencephalon. Samples are normalized to loading control GAPDH and fold induction is relative to control (n=5). (**D**) Schematic representation of the 5´ region of the *Gli3* gene. The 1.9 kb promoter region isolated and cloned into the pGL4.24 vector containing 3 putative E2f binding sites using Dcode/Mulan software. (**E**) Dual-Glo luciferase promoter assays in HEK293T cells performed with a firefly construct utilizing the *Gli3* promoter shows that E2f4 activates the *Gli3* promoter. When cells are co-transfected with Rb and E2f4 or p107 and E2f4, the activation is repressed and luciferase levels return to that of promoter alone (n=6). (**C**) Student T-test was performed relative to control and (**E**) One-way ANOVA with Tukey's was performed to compare luciferase activity. Error bars represent SEM. $^{*}p<0.05$, $^{***}p<0.001$.

from the telencephalon of *E2f4*$^{-/-}$ mutants and WT littermates. In the absence of E2f4, we detected a significant 1.7-fold increase in *Gli3* mRNA transcript (Figure 6.15C).

We next asked whether the increase in *Gli3* mRNA observed in E2f4 mutants resulted from direct regulation of the *Gli3* promoter activity by E2f4. To determine if the E2f consensus sites in the *Gli3* promoter are functionally responsive to E2f4, we created a reporter construct in which the 1.9-kb regulatory region of the *Gli3* promoter encompassing consensus E2fs site was ligated to the luciferase gene (Figure 6.15D). Transfection of E2f4 triggered a 10-fold increase in luciferase activity (Figure 6.15E). Although E2f4 is a repressor E2f, its repressive activity is dependent on pocket proteins, so that transfection of "free" E2f4 leads to increased promoter activity (Li, Hu et al. 1997, Lee, Bhinge et al. 2011). Transfection with pRb or p107 expression plasmids together with E2f4 led to repression of this E2f4-mediated activation (Figure 6.15F). These results demonstrate that E2f4 specifically binds the *Gli3* promoter, which encompasses multiple E2fs consensus sites, and that the E2f4 repressive complex acts to repress E2f-mediated activation of the *Gli3* promoter. Altogether, this suggests that E2f4 negatively regulates *Gli3*.

6.14. Atypical E2fs negatively regulate *Gli3* gene expression

As recent work has shown that, through regulation of Numb, Gli3 is critical in maintaining cadherin-mediated adhesion of the ependymal cell barrier (Wang, Ge et al. 2011), we studied the role of *Gli3*, and consequently the Shh pathway, in the *E2f7*/*E2f8* mutants. We have previously shown that Numb/Numbl and cadherins are increased in the VZ/SVZ of the compound mutants (Figure 6.14). Since E2f4 can mediate repression of

Gli3, and many of the E2fs share the same E2f consensus sites, it is possible that atypical E2fs repress *Gli3* in ependymal cells. Therefore, we hypothesize that an impaired ependymal cell barrier could result from altered *Gli3* expression in the compound mutants. To understand whether atypical E2fs can repress activity of the *Gli3* promoter, we conducted *in vitro* luciferase reporter assays in HEK293T cells using the same experimental paradigm as above (Figure 6.15D). The *Gli3* reporter construct was co-transfected with combinations of expression constructs for E2f7, E2f8 and E2f1. Our results revealed that E2f7 in particular but not E2f8 is sufficient to repress *Gli3* basal promoter activity (Figure 6.16A). Furthermore, transfection of activator E2f (E2f1) with *Gli3* triggers a strong 90-fold increase in luciferase activity above *Gli3* promoter activity. Interestingly, co-transfection with E2f1 and either E2f7 or a combination of E2f7 and E2f8 expression plasmids, leads to repression of E2f1-mediated activation of the *Gli3* promoter, which suggests that E2f7 and E2f1 counteract each other through a common mechanism. These *in vitro* results show that specifically E2f7 represses E2f-mediated activation of the *Gli3* promoter through interaction in the 5´ regulator region and it raises the possibility that E2f7 may be potent novel repressor of *Gli3* promoter activity.

Given that atypical E2fs can repress *Gli3* promoter activity, we asked whether *Gli3* mRNA was increased in the compound mutants. We performed qRT-PCR on tissue extracted from the SVZ of P3 cDKO and the control littermates. Surprisingly, quantification revealed no differences in levels of the *Gli3* transcript, although we did detect a significant 45% decrease in the levels of *Shh* mRNA in the compound mutants (Figure 6.16B). To determine, whether *Gli3* mRNA is increased specifically in ependymal cells in the compound mutants, we performed *in situ* hybridization (ISH) for *Gli3* in

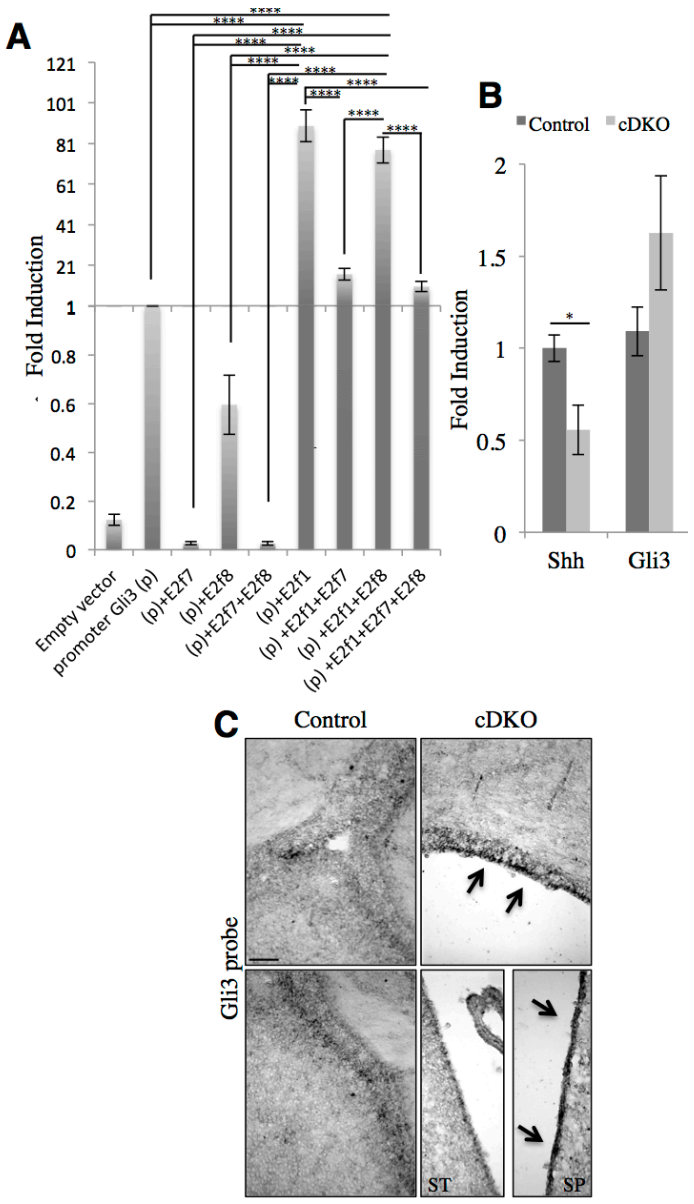

Figure 6.16: *Gli3* deregulation in cDKO mice.

(A) Dual-Glo luciferase promoter assays in HEK293T cells performed with a firefly construct utilizing *Gli3* promoter shows E2f7 and E2f7/E2f8 repress *Gli3* promoter. When cells are co-transfected with E2f1, E2f7 and E2f7/E2f8, the activity is repressed (n=3).). **(B)** Quantitative real-time PCR analysis of *Shh* and *Gli3* mRNA expression in control and cDKO at P3 tissue collected from the SVZ. Samples are normalized to loading control GAPDH and fold induction is represented by control, *Shh* expression (n=5) for both genotype and *Gli3* (n=7) for control and (n=6) for cDKO. **(C)** *In Situ* hybridization P3 control and cDKO of *Gli3* mRNA (n=4). (**A**) One-way ANOVA with Tukey's was performed to compare luciferase activity and (**B**) Student T-test was performed relative to control and Scale bar 50 μm. Error bars represent SEM. $^{*}p<0.05$, $^{****}p<0.0001$. ST=Striatum, SP=Septum.

E2f7/E2f8 mutants and the control littermates at P3. ISH analysis revealed a pronounced increase in the staining intensity in cells lining the wall of the lateral ventricle in the compound mutants, compared to control mice, indicating an increase in *Gli3* mRNA in these cells in the absence of atypical E2fs (Figure 6.16C). Because the increase in *Gli3* mRNA appears to be specific to the ependymal cell layer, this increase in total protein would be diluted when mRNA was extracted from whole SVZ dissected tissue, which probably explains why no difference in *Gli3* mRNA was observed using qPCR (Figure 6.16B). These results demonstrate that atypical E2fs, specifically E2f7, can repress *Gli3* promoter activity and in the absence of atypical E2fs, ependymal cells lining the wall of the lateral ventricle revealed an increase in the *Gli3* transcript.

In summary, our findings implicate a key role of atypical E2fs in the formation of the ependymal cell barrier. This thesis identifies a novel link between atypical E2fs and the Shh pathway gene, *Gli3*. Our results suggest that increased Gli3 in ependymal cells, would mediate downstream responses, including increase in Numb/NumbL and thus triggering accumulation of cadherin mediated adhesion of the ependyma. Dysfunction in adhesion due to accumulation of cadherin may be associated with impaired ependymal cell differentiation, proliferation and motile cilia organization. Furthermore, this also demonstrated an increase in apoptosis and gliosis. We provide new molecular evidence showing how atypical E2fs could regulate key aspects of the ependymal cell barrier, which could in turn provide insight into the underlying causes of congenital ventriculomegaly.

7. DISCUSSION

The present study demonstrates for the first time that cell cycle proteins, atypical E2fs, are essential in the regulation and maintenance of the ependymal cell barrier. Previously, ependymal cells were thought to function as a physical barrier separating the CSF from the brain parenchyma. In recent years, it has become apparent that the function of ependymal cells is also important for the development and physiology of the SVZ neural stem cell niche. Alteration of the ependymal cell barrier leads to physiological abnormalities associated with ventriculomegaly, which is considered a brain disorder where surgical intervention is unable to resolve most aspects of the disease (Jones and Klinge 2008). As little is known regarding the signalling mechanisms regulating ependymal cell differentiation, new knowledge of the physiological processes connected to ependymal cell development and integrity is important to further our understanding of the aetiology of neurodevelopmental disorders, such as ventriculomegaly.

The results of my thesis support a number of conclusions: First, I show that atypical E2fs play a crucial role in the development of the ependymal cell barrier of the lateral ventricle, and that loss of atypical E2fs disrupts ependymogenesis and leads to ventriculomegaly (Figure 7.1). Second, I show that atypical E2Fs are required for the proper regulation of *Gli3* and the downstream signalling cascade including Numb, NumbL, and the N-and E-cadherins in the lateral ventricle where striking molecular changes in this pathway take place in response to atypical E2fs deletion. These changes are highly relevant to ependymal development, as it has been shown that Gli3 plays a key

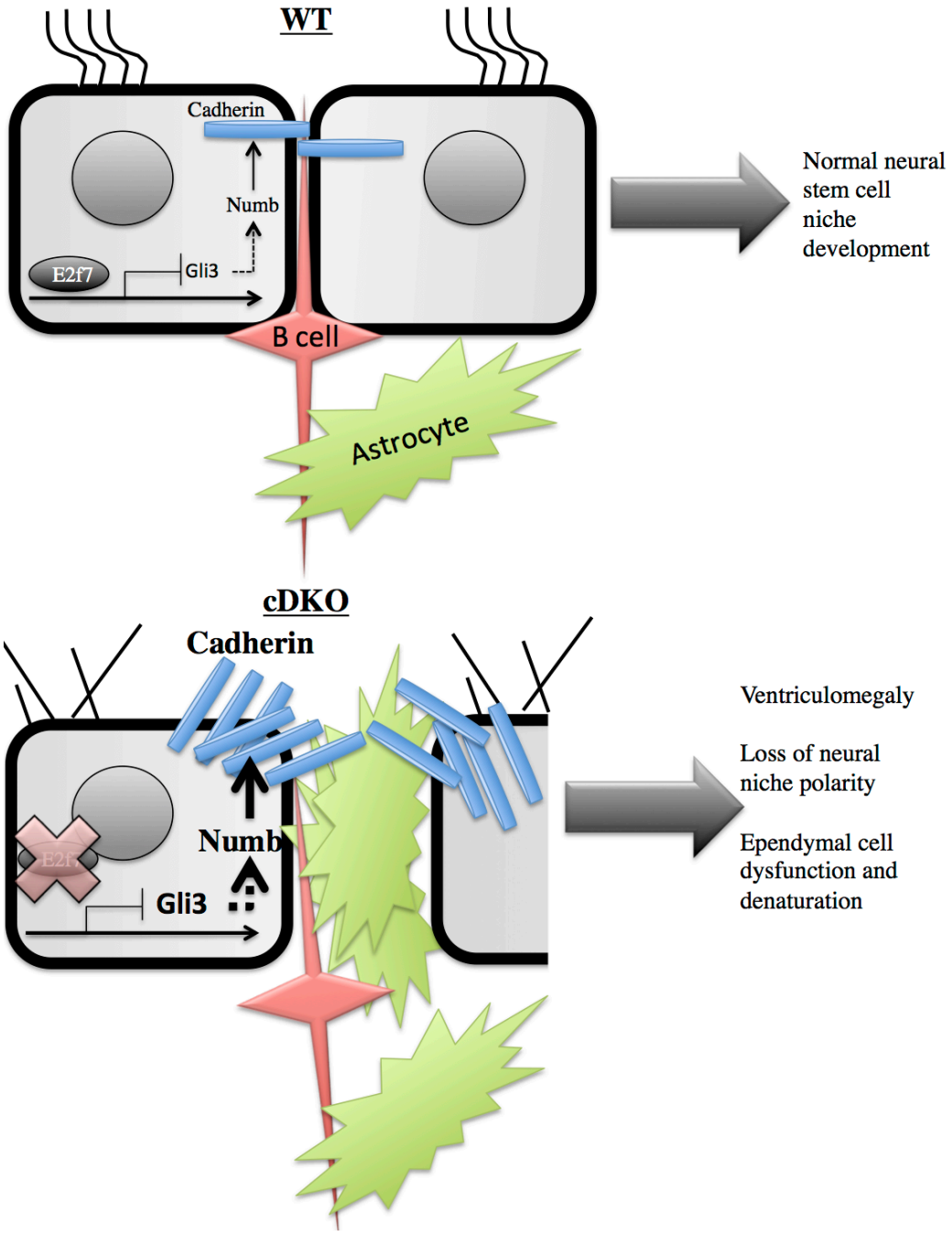

Figure 7.1: Schematic of atypical E2f regulation.

(**A**) In wild-type condition, E2f7 inhibits *Gli3* transcription, allowing regulation of the Numb pathway leading to localization of cadherins to the apical surface of the VZ. This process permits proper maturation and organization of the ependymal cell layer. (**B**) In the absence of *E27*, *Gli3* is unregulated, thereby activating the Numb pathway which leads to increase cadherins at the apical surface of the VZ. Due to the disorganization of cadherins, ependymal cells are unable to form properly leading to denaturation of the cells lining the wall of the LV, causing ventriculomegaly.

role in the maintenance of the ependymal cell barrier through the regulation of Numb (Wang, Kane et al. 2014). Interestingly, in the absence of *E2f7* and *E2f8*, we found up-regulation of Numb and NumbL protein levels in dissected SVZ tissue. While Numb mediates intracellular localization of E-cadherins and affects adherens junctions between ependymal cells (Rasin, Gazula et al. 2007), we show that loss of atypical E2fs results in increased expression of N- and E-cadherin proteins in the SVZ, demonstrating the importance of E2f7 and E2f8 in the regulation of this pathway. Third, we show that damage within the ventricular wall of the CNS results in gliosis, postnatal apoptosis and ectopic proliferation. Our results strongly suggest a model whereby atypical E2Fs play an important role in the regulation of *Gli3* and its downstream targets during ependymal cell development. **This thesis serves to support the hypothesis that atypical E2fs play a critical role in SVZ development and more specifically in the maintenance of the ependymal cell barrier.** As little was known regarding the function of atypical E2fs in the CNS, these results have implications on the basic regulation of ependymal cell development and maintenance, and further provide new insight into ependymal-associated neurodevelopmental disorders.

7.1. Role of atypical E2fs in the regulation of Gli3

Ependymal and neural progenitor cells share common precursors (Spassky, Merkle et al. 2005); however, not much is known regarding the mechanism by which RGCs differentiate into ependymal cells. Recent results indicate that the decision between ependymal cell fate versus neural progenitor fate acquisition, from RGC precursors, is specified through subtle changes in the activity of key signaling pathways.

For example, evidence suggests that differential regulation of the Shh signalling pathway, specifically *Gli3*, is involved in controlling the decision to generate ependymal versus neural precursor cells (Wang, Kane et al. 2014). The upstream mechanisms that regulate Shh-Gli signalling during ependymal cell development remain poorly understood. Here, we provide a mechanistic perspective on this process, showing that in the absence of atypical E2fs, postnatal Gli3 signalling is altered (Figure 7.1). The question as to whether atypical E2fs regulate *Gli3* expression in ependymal cells was addressed in this thesis. Previously, it has been shown that atypical E2fs bind DNA at E2f consensus sites (Westendorp, Mokry et al. 2012). Through ChIP-on-Chip and DNA sequence analysis, we have identified an E2f consensus site in the 5´ regulatory region of the *Gli3* promoter. Atypical E2fs have been shown to act as repressors of a subset of E2f target genes (Li, Ran et al. 2008, Ouseph, Li et al. 2012, Westendorp, Mokry et al. 2012). Through promoter analysis, we demonstrated that the *Gli3* promoter is responsive to E2f *in vitro*, and further show that atypical E2fs can repress *Gli3* promoter activation. Consistent with this finding, we observed an increase in *Gli3* expression in cells lining the wall of the LV in cDKO mutants. We did not detect changes in *Gli3* transcript in cDKO dissected SVZ tissue; however, this discrepancy is most likely attributable to the very small proportion of ependymal cells that comprise the dissected tissue. While Gli3 is a negative regulator of Shh signalling, we did detect a decrease in *Shh* mRNA levels by qRT-PCR in cDKO mutants, consistent with the increased *Gli3* expression detected by *in situ* hybridization. Consistent with the negative regulation of *Gli3* by atypical E2Fs, we also observe increased expression of the components of the ependymal cell Gli3-dependent signalling pathway. Specifically, we have shown increased expression of Numb and NumbL, and

increased expression of N- and E-cadherin protein levels in *E2f7*/*E2f8* mutants. Whether atypical E2fs repress *Gli3* expression directly or indirectly remains to be determined. Based on these studies, we propose that atypical E2fs are required for proper N- and E-cadherin expression through control of *Gli3*-mediated regulation of Numb gene expression. We note that Gli3 is likely one of many factors contributing to atypical E2fs-mediated ventriculomegaly. Ventriculomegaly is a complex phenomenon involving multiple factors and signalling pathways. As presented in this thesis, our results provide strong evidence that, by negatively regulating *Gli3* expression, atypical E2fs are important in the development and maintenance of the ependymal cell barrier.

7.2. Role of atypical E2fs in E2f mediated feedback loop mechanism

Conditional compound mutants of atypical E2fs allowed us to study their role specifically in the CNS. Our results suggest that atypical E2fs are critical for the formation of the ependymal cell barrier and thus, loss leads to congenital ventriculomegaly. It has long been assumed that the E2F family of transcription factors, share overlapping and redundant functional roles in tissue development and maintenance. For example, single deletion of *E2f7* or *E2f8* has little effect on mouse development, while combined deletion of the two genes leads to mid-gestational lethality (Li, Ran et al. 2008). Studies have also exposed two branches of the Rb/E2f pathway, the canonical activator E2fs (E2f1-3) and the atypical E2fs (E2f7-8), which can function to differentially regulate the same set of genes (Li, Ran et al. 2008, Chen, Ouseph et al. 2012, Ouseph, Li et al. 2012). It is believed that repressing atypical E2fs maintains the balance of the canonical activator E2fs through a feedback loop mechanism, thus

controlling the expression of activator E2fs in a cell-cycle dependent manner. This is exemplified by the observation that loss of atypical E2fs in extra-embryonic tissue leads to increased expression and activity of the canonical activator E2fs. Additionally deleting *E2F1* or *E2F3* to make triple knockout *E2f7*$^{-/-}$*;E2f8*$^{-/-}$*;E2f1*$^{-/-}$ or *E2f7*$^{-/-}$*;E2f8*$^{-/-}$*;E2f3a*$^{-/-}$ has been previously shown to rescue the phenotype caused by gene deregulation in the absence of atypical E2fs, by removing the runaway activator E2f activity (Li, Ran et al. 2008, Ouseph, Li et al. 2012). However, the cross talk between canonical activator and atypical E2fs is tissue-specific and it is unknown whether this same mechanism is present in the developing brain. In an attempt to determine whether disrupting the canonical activator and atypical E2fs feedback loop mechanism caused ventriculomegaly, we deleted *E2f1* or *E2f3* in the cDKO mutants. Neither *E2f1* nor *E2f3* deletion was sufficient to rescue congenital ventriculomegaly. Work in the retina show that activating E2fs induces apoptosis in a cell type-specific manner (Chen, Opavsky et al. 2007, Chen, Pacal et al. 2009, Chen, Chen et al. 2013). Therefore, we cannot completely exclude the possibility that defect in ependymal cell is caused by a molecular mechanism independent of the canonical activator E2fs.

In addition to the role of Rb/E2f pathway members in the control of cell proliferation, previous findings demonstrate key roles beyond cell cycle regulation. During embryonic neurogenesis, the Shh pathway is not only essential for the development of ventral structures of the telencephalon, but is also important in the maintenance and proliferation of neural precursor cells (Marti and Bovolenta 2002, Machold, Hayashi et al. 2003, Huang, Liu et al. 2010). Our previous work revealed that cell cycle regulatory protein E2f4 is required for embryonic ventral telencephalon

development (Ruzhynsky, McClellan et al. 2007). The mechanism underlying the ventral defect observed in *E2f4*-deficient embryos is associated with reduction in Shh. Gli3 is a negative regulator of Shh signalling, and thus *Gli3* haploinsufficient mice were able to rescue ventral defects in *E2f4* deficient embryo (Ruzhynsky, McClellan et al. 2007). Because E2f4 has been shown to regulate *Gli3* in embryonic neural precursor cells, and these cells appeared unaffected by the loss of atypical E2fs, we propose that atypical E2fs are not required for embryonic neurogenesis, but instead become critical postnatally for the regulation of *Gli3* in ependymal cells. These findings suggest a specific role for atypical E2fs in the formation of the ependymal cell wall; however, whether atypical E2fs function independently of the canonical Rb/E2f pathway remains to be elucidated.

7.3. Remodelling of the ependymal cell barrier

The ependyma is a single layer of multiciliated cuboidal cells, which provides a barrier between brain parenchyma and ventricular CSF, but also functions as filtration system to generate laminar flow of CSF at the surface of VZ. Loss of ependymal cells is associated with gliosis and can be linked to ventricular expansion due to aging or injury (Naureen, Waheed et al. 2013, Capilla-Gonzalez, Cebrian-Silla et al. 2014, Shook, Lennington et al. 2014). Previous work has suggested that gliosis functions to replace ependymal cells and re-establish the physical integrity of the ventricular wall, and thus promoting local repair and remodelling of the VZ (Sival, Guerra et al. 2011, Shook, Lennington et al. 2014). GFAP is expressed in mature astrocytes (Bartosik-Psujek and Stelmasiak 2001, Malmestrom, Haghighi et al. 2003) and RGCs (Roessmann, Velasco et al. 1980). It has become apparent that ependymal damage and gliosis is associated with

human and rodent models of congenital ventriculomegaly (Naureen, Waheed et al. 2013). In deleting atypical E2fs, we found the presence of gliosis and RGCs within the ventricular wall. This phenotype could result from several possible causes. One possibility is that the phenotype observed in cDKO mice is due to an underlying defect in ependymal cell differentiation. Our findings showed a decrease in S100-beta^{+} cells both *in vitro* and *in vivo*. We also found ependymal/radial glia cells populating the ventricular wall in the *E2f7*/*E2f8* conditional mutant brains. However, we did not detect changes in FoxJ1, a transcription factor required for ependymal cell differentiation (Jacquet, Salinas-Mondragon et al. 2009), nor in the ability of ependymal cells to form multiciliated cells. We therefore do not believe that a delay in ependymal cell differentiation is the underlying cause of gliosis or congenital ventriculomegaly.

Another possibility is that type-B1 cells are responding to ependymal cell denudation by differentiating into ependymal-type cells and astrocytes in an attempt to re-establish the physical integrity of the ventricular wall. This hypothesis is supported by the decrease in S100-beta cells and increased GFAP within the ependymal cell population. We also observed ectopic proliferation of ependymal cells in *E2f7*/*E2f8* mutants. Our findings were previously described in other models of ventricular damage and ventriculomegaly, where RGCs could transform into ependymal-like cells and astrocytes to mediate ventricular remodelling and local repair (Kuo, Mirzadeh et al. 2006, Luo, Shook et al. 2008, Capilla-Gonzalez, Cebrian-Silla et al. 2014).

A third possibility is that the insertion of reactive astrocytes within the ependymal cell barrier is an attempt to re-establish cellular adhesion between neighbouring cells. Our results suggest that cadherin-based adhesion is impaired in the cDKO mutants; the

VZ may undergo compensatory remodelling in an effort to maintain homeostasis of the ependymal cell wall. In animal models where adherens junction molecules are dysfunctional, VZ disruption and ventriculomegaly are common outcomes (Kuo, Mirzadeh et al. 2006, Luo, Shook et al. 2008, Wang, Kane et al. 2014). Similar to our phenotype, these animal models expressed both S100-beta and GFAP in the ependymal layer. Stemming from our findings, an attractive hypothesis is that disruption of the ependymal cell wall, due to adherens junctions' alterations in the cDKO mutants, causes compensatory VZ remodelling, where reactive astrocytes contribute to repair in an effort to maintain the integrity of the ependymal cell wall.

7.4. Biological function of atypical E2fs

It has become increasingly evident that the ependyma could play an important role in neurological and psychiatric disorders, such as autism spectrum disorder (ASD) and schizophrenia, where ventriculomegaly is a prominent feature (Lord, Cook et al. 2000, Gilmore, van Tol et al. 2001, Kempton, Stahl et al. 2010, Kotagiri, Chance et al. 2014). Human studies of ASD showed disruption of the cytoarchitecture of the ependymal cell barrier, similar to that observed in the *E2f7*/*E2f8* mutants' phenotype (Kotagiri, Chance et al. 2014). In an attempt to determine whether the compound mutants displayed psychiatric abnormalities resembling ASD, we conducted a series of behavioural tests (Appendix A). In summary, we did not detect behavioural abnormalities associated with ASD symptoms such as sociability, social memory, and communication, or with schizophrenia such as prepulse inhibition (Braff, Stone et al. 1978) in the *E2f7*/*E2f8* cDKO mutant. Furthermore, the general well-being of the mutant

mice seemed relatively normal. In conclusion, no behavioural deficits were detected as a result of the altered ependymal cell barrier and pronounced congenital ventriculomegaly in the compound mutants. Although, other behavioural abnormalities, such as repetitive behaviours (Silverman, Yang et al. 2010), seizures, anxiety and sleep disruption, which are common co-morbidities of ASD and psychiatric disorders (Crawley 2004, Chadman, Yang et al. 2009) and commonly associated with the aetiology of disorders found in human ventriculomegaly, they were not assessed in our studies, and thus, cannot be discounted.

There are several possible reasons why behavioural changes in the cDKO mutant mice were not detected. First, rodent models of ASD and other psychiatric disorders can show inconsistent and conflicting results based on the background strain of the mouse (Hessl, Dyer-Friedman et al. 2001, Spencer, Alekseyenko et al. 2011). Differences in behavioural phenotype between inbred strains of mice should to be taken into consideration. The *E2F7/E2f8* compound mutants were bred on a pure FVB/N background; although this strain is susceptible to retinal degeneration and blindness by age of weaning, FVB/N showed no strain-dependent deficit in sociability and preference to social novelty (Moy, Nadler et al. 2007). Unfortunately, there is no "perfect" strain that can be used across all behavioural paradigms. Because of its sensitivity in social behaviour, we believe that our mutant mouse on a FVB/N background was an acceptable model to test our behavioural paradigms.

Another intriguing explanation as to why we did not detect changes in cDKO mutants' behaviour could be a result of the proposed self-repair mechanism in the SVZ neural stem cell niche. Although the *E2f7/E2f8* mutants display pronounced congenital

ventriculomegaly, the presence of gliosis within the ependymal cell wall raises the possibility that the ventricular wall's integrity is maintained. Since ependymal cell wall self-repair and efficiency mechanisms, in regards to neurodevelopmental disorders, are still unclear; however, the possibility of VZ remodelling raises the question of whether gliosis is sufficient to maintain normal behavioural function. Our findings do show gliosis along the ventricular wall, and further insight on the process of gliosis on the function of the altered ependymal cell wall could shed new light on whether ependymal cells themselves play a role in neurodevelopmental psychiatric disorders. It is yet to be determined whether promoting self-repair in the ventricular wall of neurodevelopmental disorders, where ventriculomegaly is prominent, could one day be used to alleviate symptoms of psychiatric disorders such as schizophrenia and ASD.

A third possibility is the sensitivity of the behavioural tests themselves. Not much is known on the behavioural outcome due to ependymal cell defects. Other models of neurodevelopmental psychiatric disorders that include ventriculomegaly could have other underlying defects that are not present in our *E2f7*/*E2f8* mutants, which could account for their behavioural phenotype. For example, models of schizophrenia that display ventriculomegaly show impairment in amygdala-related behaviour and memory deficits due to abnormal hippocampal neurogenesis (Okun, Griffioen et al. 2010, Park, Lee et al. 2015). In ASD animal models, mutation in the cell adhesion molecule L1 is associated with intellectual disability and ventriculomegaly (Betancur, Sakurai et al. 2009). Animal models and humans with a L1 mutation also display defects in neuronal migration and a deformed corpus callosum, which can contribute to the mental retardation and epilepsy phenotype (Kenwrick, Watkins et al. 2000, Schmid and Maness

2008). Understanding the function of ependymal cells in neurodevelopmental psychiatric disorders could contribute in developing behavioural paradigms that can detect subtleties in abnormal behaviour due to ependymal cell dysfunction.

8. CONCLUSIONS AND SIGNIFICANCE OF FINDINGS

The initial aim of this study was to understand the role of atypical E2fs in the developing CNS. In doing so, we identified a novel role of atypical E2fs in development and maintenance of the ependymal cell barrier. *E2f7*/*E2f8* mutants develop congenital ventriculomegaly and display impairment in ependymal cell differentiation and motile cilia organization. Furthermore, our results demonstrate ectopic proliferation, denudation and gliosis within the ependymal cell layer. The work presented in this thesis shows a novel mechanism by which atypical E2fs are critical in maintaining the ependymal cell wall. Based on our findings, we propose that the mechanism underlying ependymal cell dysfunction lies in the increased *Gli3* expression within the ependymal cell layer leading to accumulation of cadherins at the apical surface of the VZ via the Numb pathway. In recent years, the function of ependymal cells have been shown to be important in CNS development, and dysfunction within this barrier has been linked to disorders ranging from ventriculomegaly to neuropsychiatric disorders. Knowledge of ependymal cells and the mechanisms protecting this barrier's integrity will shed new light on how disruption of these cells can cause these disease conditions.

9. FUTURE DIRECTIONS

Ventriculomegaly is present in many human pathological disorders; thus, our model is highly relevant in establishing a molecular mechanism crucial to the development and maintenance of the ependymal cell barrier structure and organization. As we were unable to rescue ventriculomegaly by deletion of *E2f1* or *E2f3*, it would be interesting to test whether the manipulation of the Gli3 or Numb pathway could rescue the phenotype observed in the cDKO mice. This could potentially be achieved by deleting a single allele of *Gli3* in the cDKO mice, as losing one allele of *Gli3* was sufficient to rescue ventral telencephalon loss in *E2f4*$^{-/-}$ animals (Ruzhynsky, McClellan et al. 2007), and atypical E2fs seem to regulate *Gli3* in a similar fashion. In addition, *Numb*/*NumbL* have been shown to be important in localization of E-cadherins (Rasin, Gazula et al. 2007). It would be intriguing to determine if loss of one allele of *Numb* and *NumbL* from cDKO mice could reinstate cadherin localization.

Alternatively, it would also need to be determined whether atypical E2fs cause ependymal cell defects in a cell autonomous manner. Since ependymal cells line the wall of the LV, injection of viral or drug treatment via CSF offers means to specifically target ependymal cells, thus minimizing off target effects. *In vivo* postnatal electroporation will permit the transfer of exogenous genes specifically to the ependymal cells during a critical time period of the SVZ neural stem cell niche development, and thus this technique can be used to selectively label ependymal cells in the VZ. Knockout of atypical E2fs in ependymal cells of newborn pups, using a Cre-vector would also offer the opportunity to study the function of atypical E2fs on a cell-to-cell basis. Furthermore, ependymal cell defects only become consistently detectable at P3, and it remains

unknown when atypical E2fs function in ependymal cell development. To truly investigate the time frame by which atypical E2fs are required for SVZ establishment, we could utilize an inducible *Nestin-Cre*^{ERT2} model, where tamoxifen would be administered throughout neural stem cell niche development: (E14.5, P0, P7, P14 and P21), and where P21 marks the beginning of the mature adult SVZ neural stem cell niche. This set of experiments will permit us to determine at which time point atypical E2fs regulates ependymal wall integrity, and thus shed new light on determining the sequence of events involved in congenital ventriculomegaly and how it relates to neurological and psychiatric disorders.

The molecular mechanism by which atypical E2fs regulate genes in the CNS is still obscure. In order to truly understand how atypical E2fs regulate *Gli3* in ependymal cells *in vivo* it will be necessary to isolate these cells for biochemical experiments. One possible means to address this question would be to specifically sort ependymal cells and perform ChIP-seq experiments using antibodies again *E2f7* and *E2f8*. This would determine if *E2F7*/*E2f8* directly bind to the *Gli3* as well as other gene promoters. One of the difficulties is that cell surface markers used for ependymal cell sorting, CD133 and CD24, are also expressed in other cell types within the SVZ. To overcome this difficulty, *FoxJ1*^{EGFP} mice (Ostrowski, Hutchins et al. 2003) could be used to conduct ChIP-seq experiments on GFP-positive sorted cells, to identify gene targets for atypical E2Fs essential for development and maintenance of ependymal cells. Future work in unraveling the gene network regulated by atypical E2fs could provide insight into the basic regulation of ependymal cells and how it may contribute to different pathologies associated with ventriculomegaly.

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11. APPENDIX A- Supplemental behavioural data

11.1. cDKO animal shows normal social interaction.

In certain human cases of autism spectrum disorder (ASD), ectopic proliferation of ependymal cells, pronounced gliosis, disorganisation of the ependymal cell barrier and mild ventriculomegaly have been observed (Kotagiri, Chance et al. 2014). Given our results, we questioned whether the *E2f7/E2f8* mutants displayed behavioural defects related to ASD. Major symptoms related to ASD include abnormal social interaction, impairment in communication and stereotypical behaviour (Lord, Cook et al. 2000, Silverman, Yang et al. 2010, Lord, Petkova et al. 2012). We first assessed the sociability and social memory of the cDKO mice by conducting a juvenile interaction behaviour test. This technique permits quantitative analysis of the time the target mouse spends interacting with a novel juvenile mouse of the same strain and gender (1st Interaction) (Figure 11.1A-C). As compared to controls, cDKO mice showed no deficits in the time spent interacting with the juvenile. Social memory is assessed 3-days later (2nd Interaction), when the target mouse is re-exposed to the same juvenile (Figure 11.1A-C). Males and females were analysed separately as the mean interaction time differed significantly from each other. Both female genotypes and male cDKO mice showed a significant decrease in the time spent interacting with the juvenile during their second encounter, demonstrating that compound mutants have the ability to recognise the familiar juvenile. To further investigate sociability behaviour in the compound mutants, we conducted a adult social interaction test. The target mouse is placed in an open field box containing an empty cage and left to explore. A novel adult mouse of the same strain and gender is introduced into the cage. Sociability is quantified by the amount of time

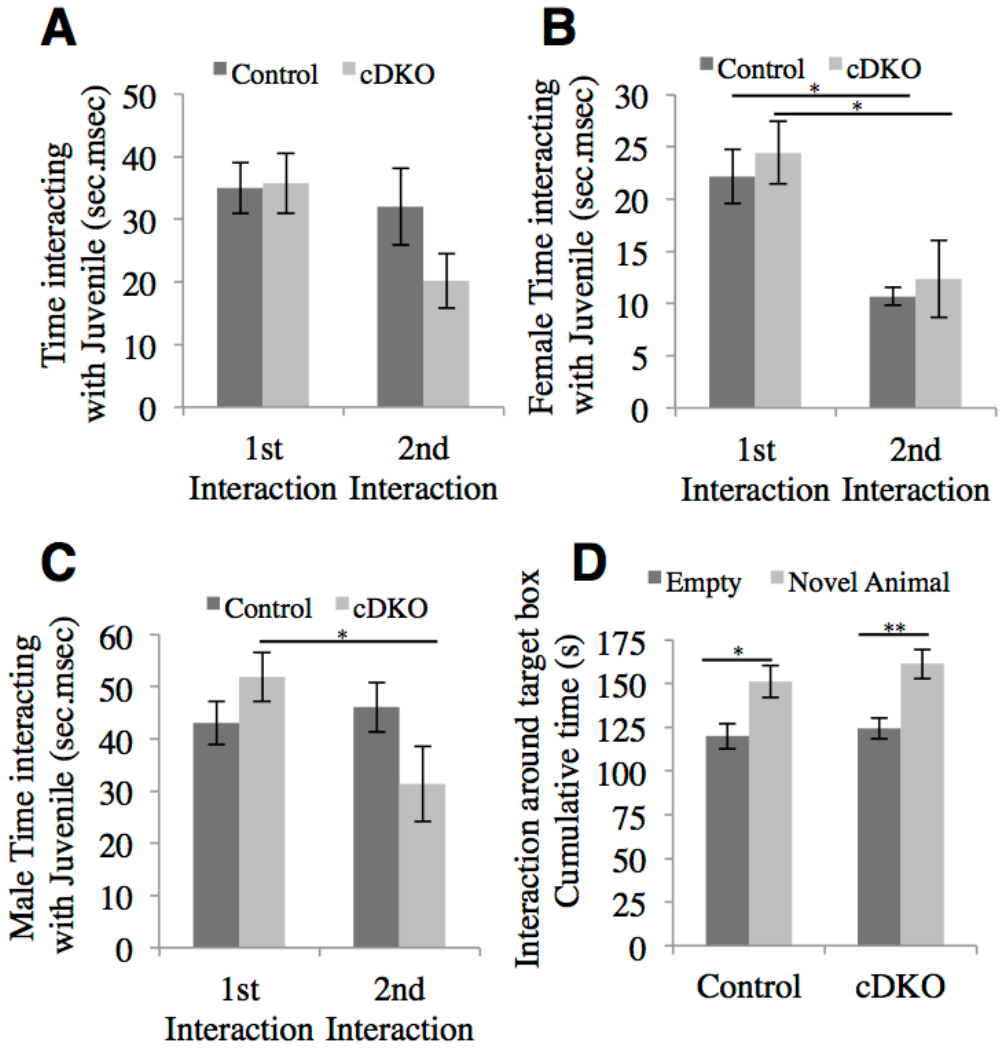

Figure 11.1: The cDKO mutants have normal social interaction and social memory

(**A-C**) Juvenile interacting test at day1 (1st Interaction), control (n=11) and mutant (n=11) mice spent a similar amount of time interacting with juvenile mice of the same sex and strain. (**B**) Control (n=5) and mutant (n=7) female mice significantly reduced their interaction time with juvenile mice and (**C**) Male control (n=6) mice did not decrease their interaction time with the same juvenile 3 days later (2nd interaction), however, male mutant (n=4)

(**D**) Adult social interaction test shows, when exposed to inanimate target and then to a caged social target, both control (n=11) and cDKO mutant (n=9) showed normal preference for social target over inanimate target.

Error bars represent SEM. $^{*}p<0.05$, $^{**}p<0.01$.

the target mouse interacts with the cage with or without the novel mouse. Both groups significantly preferred interaction with the cage containing the novel animal (Figure 11.1D). These results demonstrate that loss of atypical E2fs does not appear to influence sociability or social memory.

11.2. cDKO animal display normal social communication behaviour.

We next asked whether loss of atypical E2fs could lead to communication deficits. To assess this question, we used an olfactory social odours test, olfactory communication is quantified by the amount of time the target animal spends sniffing a cotton swab of a specific scent. In a described mouse model of ASD, the ability of mice to distinguish between non-social and social odours is impaired (Crawley 2004). As compared to control, the cDKO group, preferred sniffing the cotton swab containing the scent of an unfamiliar male (Social M) or female (Social F), then with a non-social odour such as water, almond or banana (Figure 11.2A), suggesting that compound mutants do not exhibit olfactory communication deficits.

Another mean of communication is through ultrasonic vocalization (USV). When removed from their mother, newborn pups from a genetic model of ASD, show USV deficits (Shu, Cho et al. 2005, Panksepp, Jochman et al. 2007, Scattoni, Ricceri et al. 2011, Wohr, Roullet et al. 2011). To determine whether the compound mutants have altered USV, we separated 8-day old (P8) pups from their mother and recoded the number of cries (event) and total time of cries using four probes simultaneously recording at 50, 60, 70 and 80 Hz (Figure 11.2B&C). The same pup was recorded using the same procedure 2 days later (P10) (Figure 11.2C&E). The *E2f7*/*E2f8* mutant did not differ

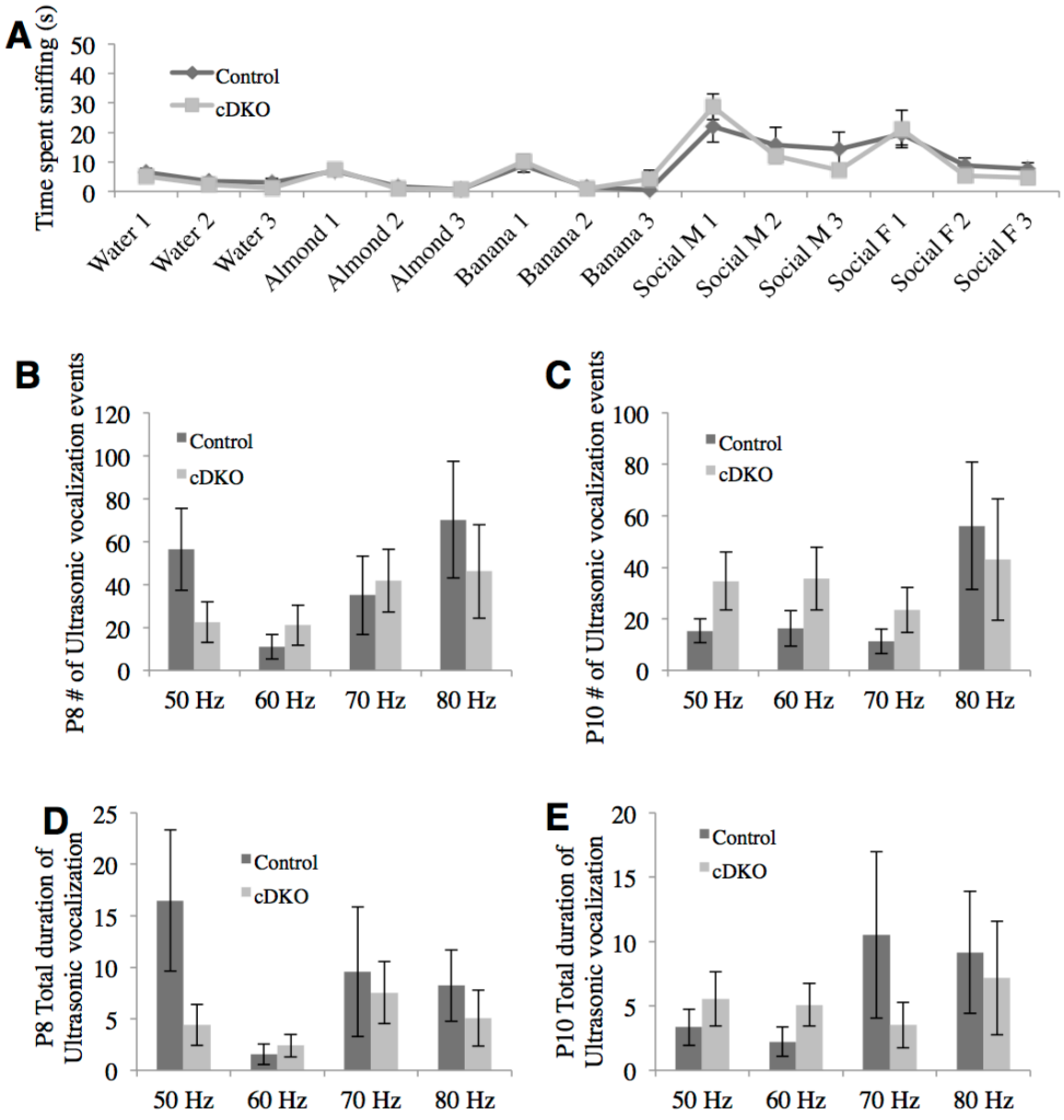

Figure 11.2: Social communication was largely normal in cDKO mice.

(**A**) Olfactory habituation/dishabituation shows that both control (n=12) and mutants (n=11) mice can smell non-social and social odors. After the first presentation of each non-social odor, water, almond and banana, both groups show decrease sniffing response (habituation). Both group also elicit an increase sniffing response after each presentation of a new smell (dishabituation), with higher preference for social odors.
Number of ultrasonic vocalization in (**B**) P8 and 2 days later (**C**) P10 old mice separated from the dame is unchanged between control (n=9) and mutant (n=9) at all 4 frequencies tested. Total duration of the ultrasonic vocalization call is similar at (**D**) P8 and (**E**) P10 in both the control and cDKO mice.
Error bars represent SEM.

from control at both P8 and P10, suggesting that loss of atypical E2fs does not lead to alter USV. Taken together, these results indicate that cDKO mice do not exhibit discernable social communication behavioural deficits.

11.3. Nesting and sensory gating behaviour are normal in cDKO mice.

While the cDKO animals do not display any core social behavioural abnormalities associated with ASD, other psychiatric disorder such as schizophrenia are associated with progressive enlargement of the lateral ventricle (Kempton, Stahl et al. 2010). Therefore, we next asked whether mutants had behavioural phenotypes indicative of other neuropsychiatric disorders. To address this question, we used the prepulse inhibition (PPI) test, which measures sensorimotor gating in mice. Both human patients and animal models of schizophrenia display diminish PPI (Braff, Stone et al. 1978). The percentage of PPI increased with increased prepulse intensity and the percentage of PPI level was comparable between both genotypes (Figure 11.3A), suggesting that cDKO mice exhibit normal sensorimotor gating functions.

We then assessed the well being of the cDKO mice, using a nesting behaviour test. Deterioration of nesting ability has been used as a parameter for neurodegenerative disease such as Alzheimer and Parkinson disease as well as psychiatric disorders such as schizophrenia, ASD and obsessive-compulsive disorder (Deacon 2012, Jirkof 2014). Nest quality was scored on a five-point scale where 1 represents an untouched nestlet and 5 represent a near perfect nest (Deacon 2012). No significant difference in nest quality was observed between the cDKO mice compared to controls (Figure 11.3B). This behavioural assessment indicates that cDKO mice have a good general health.

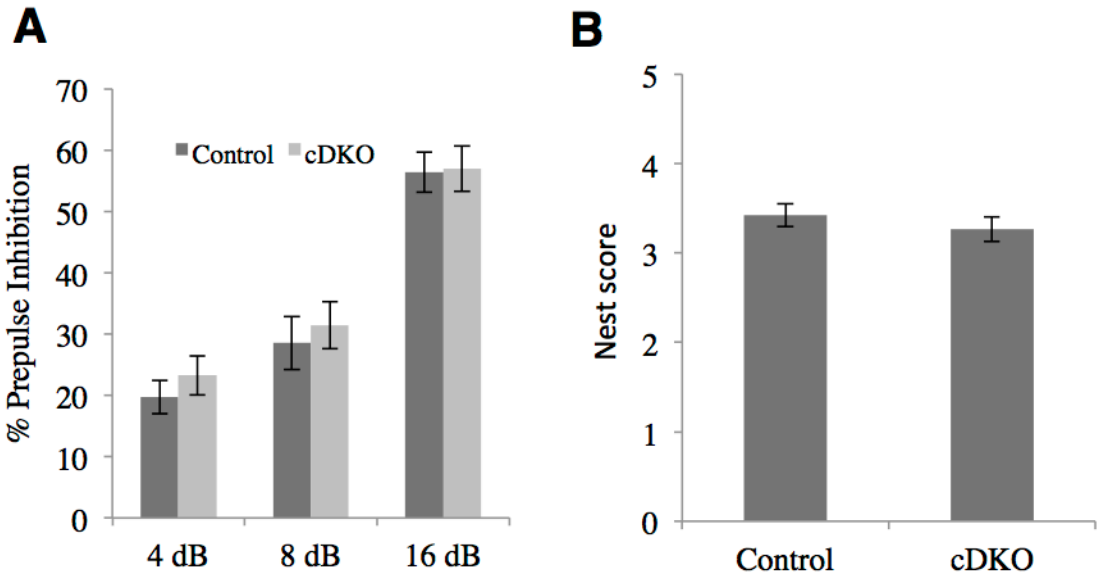

Figure 11.3: cDKO mice show normal nesting behaviour and prepulse inhibition of acoustic intensity.

(**A**) Nesting behavioural test, shows no differences in nest scores for control (n=28) and cDKO (n=21) animal. (**B**) Distribution for prepulse inhibition of acoustic startle response with 4, 8 and 16 dB prepulse stimulus for cDKO (n=11) and control (n=10) shows no differences. Data represent % of prepulse inhibition. Error bars represent SEM.

In summary, ASD and schizophrenia are a complex neurodevelopmental disorder containing multiple molecular facets ranging from changes in synaptic development, signalling, transcription and epigenetics. Although, we did not detect behavioural abnormalities associate with sociability, social memory, and communication in the cDKO mutants. Sensorimotor gating and the well-being of the mutant mice seemed relatively unaffected. So far, the *E2f7*/*E2f8* mutants display altered ependymal cell barrier and pronounced congenital ventriculomegaly, and although, the behavioural phenotype of these mutants do not fit criteria of ASD, we cannot disregard other behavioural abnormalities that could be associated with the aetiology of disorders associated with ventriculomegaly.

12. APPENDIX B- Curriculum Vitae

Delphie Dugal-Tessier

EDUCATION

Degree in progress:

Ph.D.	Program: Neuroscience Department: Cellular and Molecular Medicine University of Ottawa Ottawa, Ontario, Canada Thesis Supervisor: Dr. Ruth Slack

Degree held:

B.A.	Program: Psychology Department: Social Science University of Ottawa Ottawa, Ontario, Canada Thesis Supervisor: Dr. Philip Firestone

WORK EXPERIRENCE

2010-present	Graduate student (Ph.D), Department of Cellular and Molecular Medicine, Neuroscience program, University of Ottawa
2008-2010	Graduate student (MSc transferred to Ph.D), Department of Cellular and Molecular Medicine, Neuroscience program, University of Ottawa
2008-2012	Research assistant in child depression study, University of Ottawa Institute of Mental Heath Research – Royal Ottawa Health Care group. Project supervisor: Dr. Martine Flament
2008-2009	Research assistant in adult depression study, University of Ottawa Institute of Mental Heath Research – Royal Ottawa Health Care group. Project supervisor: Dr. Pierre Blier
2007	Research student, treatment and evaluation of adult sexual offenders, Rockwood Psychological Services, Kingston, Ontario, Project supervisor: Dr. William Marshall

SCHOALRY AND PROFESSIONAL ACTIVITIES

2012-2013	President, Cellular and Molecular Medicine/Neuroscience (CMM/NSC) Student's Council (University of Ottawa
2011-2012	VP in communication, CMM/NSC Student's Council (University of Ottawa)
2009-2011	VP social and athletic, CMM/NSC Student's Council (University of Ottawa)
2013	CMM/NSC Representative for student run Faculty Seminar Series- invited and hosted Dr. Victor Ambros
2012	Student Speaker on stress management- Professionalism and Professional Skills class, University of Ottawa, Faculty of Medicine
2008-2012	CMM/NSC Representative for University of Ottawa Institute of Mental Heath Research-Young Researchers Forum
2010-2012	Science Activity Demonstrator, Let's Talk Science (university of Ottawa)

AWARDS AND SCHOLARSHIPS

2014	**NeuroDevNet-ISDN Conference Travel Award** International Society for Developmental Neuroscience of Montreal, 1000$
2013	**5th Annual Brain Health Research Day- Trainee Award Winner** University of Ottawa, Faculty of Medicine
2013	**Participation in the CIHR Research Poster Presentation** Canadian Student Health Research Forum, Winnipeg, Manitoba
2012	**Faculty of Medicine Award of Excellence – Leadership Award** University of Ottawa, Faculty of Medicine, 500$
2012-2014	**Ontario Graduate Scholarship (OGS)** University of Ottawa, Department of Cellular and Molecular Medicine, Neuroscience 15 000$
2011- 2012	**Queen Elizabeth II Graduate Scholarships in science and technology (QEII-GSST)** University of Ottawa, Department of Cellular and Molecular Medicine, Neuroscience 12 000$

2011 **Taichman Award-Neuroscience poster day award**
University of Ottawa, Department of Cellular and Molecular Medicine, Neuroscience
250$

2010-2011 **Ontario Graduate Scholarships in Science and Technology (OGSST)**
University of Ottawa, Department of Cellular and Molecular Medicine, Neuroscience
12 000$

2009-2010 **Ontario Graduate Scholarships in Science and Technology (OGSST)**
University of Ottawa, Department of Cellular and Molecular Medicine, Neuroscience
12 000$

2009 **Renin Award B.H.R.D. conference**
University of Ottawa, Faculty of Medicine, 100$

2008- 2010 **Graduate Admission Scholarship**
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PUBLICATIONS

Papers in refereed journals: 3
Papers in preparation: 2
Select meeting Abstracts: 15
Invited presentations: 1

Papers in Refereed Journals:

1. Julian LM, Liu Y, Pakenham CA, **Dugal-Tessier D**, Ruzhynsky V, Bae S, Tsai SY, Leone G, SlackRS*, and Blais A* (2015) Tissue-specific targeting of cell fate regulatory genes by E2f factors, *Cell Death and Differentiation*. [Epub ahead of print]

2. Andrusiak MG, McClellan KA, **Dugal-Tessier D**, Julian LM, Rodrigues S, Park , Kennedy TE and Slack RS (2011) Rb/E2F regulates expression of neogenin during neuronal migration, *Mol Cell Biol,31(2):238-47*.

3. McClelland KA, Vanderluit JL, Julian LM, Andrusiak MG, **Dugal-Tessier D**, Park DS, Slack RS (2009). p107/E2F pathway regulates FGF2 responsiveness in neural precursor cells, *Mol Cell Biol* 29(17):4701-13.

Papers in Preparation:

1. **Dugal-Tessier D**, Vandenbosch R, Khacho M, Svoboda DS, Weijts B, Park DS, Leone G, de Bruin A and Slack RS. Atypical E2fs are required for the maintenance of the ependymal cell barrier

2. Vandenbosch R, Clark AM, **Dugal-Tessier D**, Andrusiak MG, Park DS and Slack RS. The retinoblastoma protein is crucial for denate gyrus development and adult hippocampal neurogenesis

Meeting Abstracts

1. *<u>**Dugal-Tessier D**</u>, Vandenbosch R, Khacho M, Weijts B, Park DS, Leone G, de Bruin A and Slack RS. Absence of atypical E2f leads to ventriculomegaly. Brain Health Research Day, Ottawa, ON, 2014. **Invited speaker- oral presentation**

2. <u>**Dugal-Tessier D**</u>, Vandenbosch R, Khacho M, Weijts B, Park DS, Leone G, de Bruin A and Slack RS. Absence of atypical E2f leads to ventriculomegaly. International Society for Developmental Neuroscience, Montreal, QC, 2014.

3. <u>**Dugal-Tessier D**</u>, Vandenbosch R, Khacho M, Weijts B, Park DS, Leone G, de Bruin A and Slack RS. Absence of atypical E2f leads to ventriculomegaly. Third International RB Meeting, Monterey, CA, USA, 2013.

4. <u>**Dugal-Tessier D**</u>, Vandenbosch R, Khacho M, Weijts B, Park DS, Leone G, de Bruin A and Slack RS. Absence of atypical E2f leads to ventriculomegaly. CIHR Research Poster Presentation, Winnipeg, MB, 2013.

5. <u>**Dugal-Tessier D**</u>, Vandenbisch R, Khacho M, Weijts B, Park DS, Leone G, de Bruin A and Slack RS. Absence of atypical E2f leads to ventriculomegaly. Third International RB Meeting, Monterey, CA, USA, 2013.

6. <u>**Dugal-Tessier D**</u>, Andrusial MG, Julian LM, Ruzhynsky VA, Park DS and Slack RS. Rb/E2F pathway targets Shh signaling in neural precursor cells. Second International RB Meeting, Toronto, ON, 2011.

7. <u>**Dugal-Tessier D**</u>, Andrusial MG, Julian LM, Ruzhynsky VA, Park DS and Slack RS. Rb/E2F pathway targets Shh signaling in neural precursor cells. Keystone Symposia on adult neurogenesis, Taos, New Mexico, United States of America, 2011.

8. <u>**Dugal-Tessier D**</u>, Andrusial MG, Julian LM, Ruzhynsky VA, Park DS and Slack RS. Rb/E2F pathway targets Shh signaling in neural precursor cells. 3rd Annual Brain Health Research Day, Ottawa, ON, 2011.

9. **Dugal-Tessier D**, Andrusiak MG, Ruzhynsky VA, Julian LM, Park DS and Slack RS. E2F4 is essential for development of the ventral telencephalon. International Society for Developmental Neuroscience, Estoril, Portugal, 2010.

10. **Dugal-Tessier D**, Ruzhynsky VA, Julian LM, Andrusiak, MG, Park, DS, Wallace, VA and Slack RS. Cross-talk between the pRb/E2F and Shh pathways is required for development of the ventral telencephalon. First International RB Meeting, Toronto, ON, 2009.

11. **Dugal-Tessier D**, Ruzhynsky VA, Julian LM, Andrusiak, MG, Park, DS, Wallace, VA and Slack RS. Cross-talk between the pRb/E2F and Shh Pathways is Required for Development of the Ventral Telencephalon**.** First International RB Tumor Suppressor Meeting, Toronto, Canada, November 2009.

12. **Dugal-Tessier D**, Ruzhynsky VA, Julian LM, Andrusiak, MG, Park, DS, Wallace, VA and Slack RS. Cross-talk between the pRb/E2F and Shh Pathways is Required for Development of the Ventral Telencephalon. Cold Spring Harbor/Wellcome Trust Mouse Genetics Meeting, Cambridge, UK, September 2009.

13. **Dugal-Tessier D**, Ruzhynsky VA, Julian LM, Andrusiak, MG, Park, DS, Wallace, VA and Slack RS. E2F4 is essential for the development of the ventral telencephalon. 1st Annual Brain Health Research Day, Ottawa, ON, 2009.

14. **Dugal-Tessier D**, Ruzhynsky VA, Julian LM, Andrusiak, MG, Park, DS, Wallace, VA and Slack RS. E2F4 is essential for the development of the ventral telencephalon. University of Ottawa Institute of Mental Health Research, Young Researchers Forum Conference, Ottawa, ON, 2009.

15. **Dugal-Tessier D**, Ruzhynsky VA, Julian LM, Andrusiak, MG, Park, DS, Wallace, VA and Slack RS. Negative regulation of Gli3 by E2F4 is essential for the development of the ventral telencephalon. Great Lakes Mammalian Development Meeting, Toronto, ON, 2009.

12. APPENDIX C

- Co-author publication reprints – first page

MOLECULAR AND CELLULAR BIOLOGY, Sept. 2009, p. 4701–4713 Vol. 29, No. 17
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The p107/E2F Pathway Regulates Fibroblast Growth Factor 2 Responsiveness in Neural Precursor Cells[▽]

Kelly A. McClellan,† Jacqueline L. Vanderluit,†‡ Lisa M. Julian, Matthew G. Andrusiak, Delphie Dugal-Tessier, David S. Park, and Ruth S. Slack*

University of Ottawa, Department of Cellular and Molecular Medicine, 451 Smyth Rd, Ottawa, Ontario K1H 8M5, Canada

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We have previously shown that p107, a member of the retinoblastoma (Rb) cell cycle regulatory family, has a unique function in regulating the pool of neural precursor cells. As the pool of progenitors is regulated by a limiting supply of trophic factors, we asked if the Rb/E2F pathway may control the size of the progenitor population by regulating the levels of growth factors or their receptors. Here, we demonstrate that fibroblast growth factor 2 (FGF2) is aberrantly upregulated in the brains of animals lacking Rb family proteins and that the gene encoding the FGF2 ligand is directly regulated by p107 and E2F3. Chromatin immunoprecipitation assays demonstrated that E2F3 and p107 occupy E2F consensus sites on the FGF2 promoter in the context of native chromatin. To evaluate the physiological consequence of FGF2 deregulation in both p107 and E2F3 mutants, we measured neural progenitor responsiveness to growth factors. Our results demonstrate that E2F3 and p107 are each mediators of FGF2 growth factor responsiveness in neural progenitor cells. These results support a model whereby p107 regulates the pool of FGF-responsive progenitors by directly regulating FGF2 gene expression in vivo. By identifying novel roles for p107/E2F in regulating genes outside of the classical cell cycle machinery targets, we uncover a new mechanism whereby Rb/E2F mediates proliferation through regulating growth factor responsiveness.

Cell cycle genes have been found to play an important role in brain development, with numerous molecules regulating the G_1/S transition having been shown to regulate neural precursor proliferation (reviewed in reference 38). Perhaps the most important regulators of the G_1/S transition are the retinoblastoma protein (Rb) and its closely related family member p107. Rb is a pivotal regulator of neural precursor proliferation and the timing of cell cycle withdrawal. For example, Rb has been shown to regulate terminal mitosis of neuroblasts in the central and peripheral nervous systems and retina (7, 18, 34, 35). Furthermore, recent evidence has emerged indicating that Rb itself is capable of regulating diverse cellular processes in the nervous system beyond proliferation. Roles for Rb have been indicated in laminar patterning of the cortex and neuronal migration (17; reviewed in reference 38). These studies highlight the importance of Rb in regulating neural cell populations. In contrast to Rb, little is known about the role of p107. While its role was originally thought to overlap with and compensate for that of Rb (29), distinct functional differences in tissues such as muscle, chondrocytes, and adipocytes, have emerged, suggesting otherwise (10, 28, 51). We have recently shown that p107 plays a unique role, one distinct from Rb, in regulating neural precursor cell numbers in the developing and adult brain (60). p107 null neural precursor cells have an enhanced capacity for self-renewal and, consistent with this, exhibit expanded populations of both precursors and progenitors. While we have previously demonstrated that the increased self-renewal capacity and neural precursor numbers are due, in part, to an upregulation of the Notch-Hes signaling pathway (61), the mechanisms that sustain the increased population are still unknown.

The E2F family of transcription factors, comprised of E2F1 to E2F8, are key Rb/p107-interacting targets best known for their role in promoting cell cycle progression (reviewed in reference 59). Accumulating in vitro and in vivo evidence, however, suggests that E2Fs are capable of regulating expression of a broad spectrum of genes and diverse physiological processes (reviewed in reference 39). In vitro, microarray studies examining changes in gene expression in response to various models of deregulated E2F expression have each identified groups of overlapping novel target genes with well-characterized roles in differentiation, development, and migration (3, 12, 25, 33, 41, 43, 68). Chromatin immunoprecipitation (ChIP)-on-chip studies have localized E2Fs to a number of gene promoters unrelated to cell cycle (1, 2, 6, 26, 47, 64, 65). In vivo, E2Fs have been implicated in a number of distinct aspects of nervous system development. E2F4 has been shown to regulate development of the ventral telencephalon through a genetic interaction with the Sonic hedgehog pathway (50), while E2F1 and E2F3 have been implicated in mediating neural precursor proliferation (11, 37). Intriguingly, in vivo models are emerging to suggest that Rb family members interact with E2Fs to mediate novel functions in nervous system development. For example, Rb has been shown to interact with both E2F3 and E2F1 to mediate neural precursor proliferation and cell cycle exit (8, 37). Additionally, Rb has been shown to mediate neural migration and differentiation, in a manner beyond cell cycle regulation, uniquely through E2F3 (8, 37). Given the emerging

* Corresponding author. Mailing address: Department of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Rd., Ottawa, ON K1H 8M5, Canada. Phone: (613) 562-5800, ext. 8458. Fax: (613) 562-5403. E-mail: rslack@uottawa.ca.

† These authors contributed equally.

‡ Present address: Memorial University of Newfoundland, Division of BioMedical Sciences, St. John's, Newfoundland A1B 3V6, Canada.

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Rb/E2F Regulates Expression of Neogenin during Neuronal Migration▽

Matthew G. Andrusiak,[1] Kelly A. McClellan,[1] Delphie Dugal-Tessier,[1] Lisa M. Julian,[1] Sonia P. Rodrigues,[2] David S. Park,[1] Timothy E. Kennedy,[2] and Ruth S. Slack[1]*

Department of Cellular Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H 8M5,[1] and Centre for Neuronal Survival, Montreal Neurological Institute, Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec, Canada[2]

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The Rb/E2F pathway has long been appreciated for its role in regulating cell cycle progression. Emerging evidence indicates that it also influences physiological events beyond regulation of the cell cycle. We have previously described a requirement for Rb/E2F mediating neuronal migration; however, the molecular mechanisms remain unknown, making this an ideal system to identify Rb/E2F-mediated atypical gene regulation *in vivo*. Here, we report that Rb regulates the expression of *neogenin*, a gene encoding a receptor involved in cell migration and axon guidance. Rb is capable of repressing E2F-mediated *neogenin* expression while E2F3 occupies a region containing E2F consensus sites on the *neogenin* promoter in native chromatin. Absence of Rb results in aberrant neuronal migration and adhesion in response to netrin-1, a known ligand for neogenin. Increased expression of neogenin through *ex vivo* electroporation results in impaired neuronal migration similar to that detected in forebrain-specific Rb deficiency. These findings show direct regulation of *neogenin* by the Rb/E2F pathway and demonstrate that regulation of *neogenin* expression is required for neural precursor migration. These studies identify a novel mechanism through which Rb regulates transcription of a gene beyond the classical E2F targets to regulate events distinct from cell cycle progression.

The Rb pathway is best characterized for its role in regulating cell cycle progression through E2F-mediated transcriptional regulation of classical cell cycle machinery target genes. Recently, however, accumulating *in vivo* and *in vitro* evidence is emerging to suggest that Rb and E2F are capable of regulating expression of atypical target genes with functions other than cell cycle regulation in cell-type-specific manners (reviewed in reference 35). *In vivo*, several studies have emerged that implicate Rb and E2F interaction in novel processes beyond well-characterized roles in cell cycle regulation (10; for a review, see reference 6). In the nervous system, in particular, we have recently shown that an Rb-E2F3 interaction mediates migration of a subpopulation of GABAergic interneurons (34). In the same study, we also observed deregulation of a number of genes with known roles in neuronal migration in cell populations lacking Rb, suggesting a role for E2F3 in regulating transcription of novel targets (34). A second cell cycle-independent role for E2F3a in regulating Rb-mediated interneuron differentiation was also reported in the retina (9). Thus far, *in vivo* studies have failed to identify the mechanism through which these cell cycle-independent processes occur.

In parallel, *in vitro* several microarray studies examining changes in gene expression in response to various models of deregulated E2F expression have each identified groups of overlapping novel target genes with well-characterized roles in differentiation, development, and migration (5, 15, 25, 31, 39, 41, 60). More recently, chromatin immunoprecipitation (ChIP)-on-chip studies have identified putative E2F binding sites within the promoters of a number of genes unrelated to the cell cycle (3, 4, 7, 28, 46, 56, 57). Finally, by using an approach whereby novel genes induced by E2F1 are identified based on subtraction screening, genes with known roles in differentiation and migration were identified as being directly induced by E2F1 in a cell cycle-independent manner (26). Thus, these data provide evidence that our understanding of the significance of Rb/E2F function should be expanded to include transcriptional regulation of genes beyond the well-characterized subset of targets that regulate the cell cycle.

Our identification of a role for Rb/E2F3 in mediating neuronal migration represents an attractive model to identify novel cell cycle-independent E2F target genes in the context of an *in vivo* physiological function (16, 34). Given our previous observations revealing (i) deregulation of a number of genes in families of known chemotactic ligands and receptors implicated in neuronal migration in the absence of Rb; and (ii) the cell-autonomous requirement for Rb in neuronal migration, we hypothesized that Rb/E2F may modulate the transcription of novel target genes involved in neuronal migration. We focused our efforts on *neogenin*, a receptor for the netrin and repulsive guidance molecule (RGM) families of chemotropic ligands (reviewed in reference 14). Notably, neogenin is highly expressed by a subpopulation of interneurons migrating from the ventral forebrain and has been independently identified, in an *in vitro* overexpression system, as an E2F-regulated gene (26, 34). Here, we report that Rb directly regulates the expression of a nontraditional target, *neogenin*. Rb is capable of repressing E2F-mediated transcription of *neogenin* while E2F3 binds to a region containing a conserved E2F consensus site on

* Corresponding author. Mailing address: Department of Cellular Molecular Medicine, University of Ottawa, 451 Smyth Rd., Ottawa, Ontario K1H 8M5, Canada. Phone: (613) 562-5800, ext. 8458. Fax: (613) 562-5403. E-mail: rslack@uottawa.ca.

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Tissue-specific targeting of cell fate regulatory genes by E2f factors

LM Julian[1,4], Y Liu[2], CA Pakenham[1], D Dugal-Tessier[1], V Ruzhynsky[1], S Bae[3], S-Y Tsai[3], G Leone[3], RS Slack*[,1] and A Blais*[,2]

Cell cycle proteins are important regulators of diverse cell fate decisions, and in this capacity have pivotal roles in neurogenesis and brain development. The mechanisms by which cell cycle regulation is integrated with cell fate control in the brain and other tissues are poorly understood, and an outstanding question is whether the cell cycle machinery regulates fate decisions directly or instead as a secondary consequence of proliferative control. Identification of the genes targeted by E2 promoter binding factor (E2f) transcription factors, effectors of the pRb/E2f cell cycle pathway, will provide essential insights into these mechanisms. We identified the promoter regions bound by three neurogenic E2f factors in neural precursor cells in a genome-wide manner. Through bioinformatic analyses and integration of published genomic data sets we uncovered hundreds of transcriptionally active E2f-bound promoters corresponding to genes that control cell fate processes, including key transcriptional regulators and members of the Notch, fibroblast growth factor, Wnt and Tgf-β signaling pathways. We also demonstrate a striking enrichment of the CCCTC binding factor transcription factor (Ctcf) at E2f3-bound nervous system-related genes, suggesting a potential regulatory co-factor for E2f3 in controlling differentiation. Finally, we provide the first demonstration of extensive tissue specificity among E2f target genes in mammalian cells, whereby E2f3 promoter binding is well conserved between neural and muscle precursors at genes associated with cell cycle processes, but is tissue-specific at differentiation-associated genes. Our findings implicate the cell cycle pathway as a widespread regulator of cell fate genes, and suggest that E2f3 proteins control cell type-specific differentiation programs by regulating unique sets of target genes. This work significantly enhances our understanding of how the cell cycle machinery impacts cell fate and differentiation, and will importantly drive further discovery regarding the mechanisms of cell fate control and transcriptional regulation in the brain, as well as in other tissues.
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The classical cell cycle regulatory pRb/E2f pathway has emerged as an important effector of fate decisions in a number of cell types, including in the brain. Cell cycle dynamics strongly influence neural precursor cell (NPC) maintenance and neurogenesis,[1–4] and gain- or loss-of-function studies have demonstrated key roles for cell cycle proteins, including the E2f family, in NPC fate decisions.[3–16] E2f3 is required for proper cortical migration of neurons and to maintain the balance between NPC self-renewal, proliferation and differentiation, and its loss disrupts long-term neurogenesis and cortical function; E2f1 deficiency impairs NPC proliferation, and E2f4 deficiency leads to inhibition of NPC self-renewal and severe defects in telencephalic development.[6,8–10,17]

A pivotal question is whether cell fate control by the pRb/E2f pathway is largely a consequence of cell cycle regulation, or due to direct regulation of cell fate-associated genes. We recently found that loss of E2f3a and E2f3b leads to opposing defects in NPC maintenance and differentiation.[8] The fact that this occurred without affecting cell cycle dynamics strongly suggests that fate control by E2fs is not secondary to cell cycle regulation. In addition, a number of key cell fate genes and pathways have been identified as E2f-regulated targets driving E2f-dependent fate decisions in NPCs. These include the neurogenesis and migration genes *Dlx1/Dxl2* and *Neo1* (Neogenin),[18,19] the growth factor fibroblast growth factor 2 (*Fgf2*),[17] the pluripotency and self-renewal factor *Sox2,*[8] and the Notch/Hes[11] and Sonic Hedgehog pathways.[10] Together, these findings demonstrate a direct role for pRb/E2f at cell fate-associated genes, but the extent of this interaction is unknown. Many of these studies focused on single pRb or E2f factor knock-out models, and because E2fs exhibit extensive redundancy in their biological functions and genomic binding patterns,[20,21] it is likely that more E2f target genes relevant to cell fate exist than those that have been reported. Understanding the full regulatory potential of the cell cycle machinery in the brain therefore necessitates an appreciation

[1]Neuroscience program, Department of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada; [2]Department of Biochemsitry, Microbiology and Immunology, Ottawa Institute of Systems Biology, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada and [3]Solid Tumor Biology Program, Department of Molecular Virology, Immunology, and Medical Genetics and Department of Molecular Genetics, Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, USA

*Corresponding author: A Blais, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada. Tel: +613 562 5800 extn. 8253; Fax: +613 562 5452; E-mail: Alexandre.Blais@uottawa.ca

or RS Slack, Department of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada. Tel: +613 562 5800 extn. 8458; Fax: +613 562 5403; E-mail: rslack@uottawa.ca

[4]Current address: Sprott Centre for Stem Cell Research, Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, ON, Canada.

Abbreviations: ChIP, chromatin immunoprecipitation; Ctcf, CCCTC binding factor; E (ie. E14.5), embryonic day; E2f, E2 promoter binding factor; Fgf, fibroblast growth factor; GO, gene ontology; MB, myoblast; NPC, neural precursor cell; TSS, transcriptional start site; WT, wild-type

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