Regulation of Neural Precursor Cell Fate by the E2f3a and E2f3b Transcription Factors

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ABSTRACT

The classical cell cycle regulatory pathway is well appreciated as a key regulator of cell fate determination during neurogenesis; however, the extent of pRB/E2F function in neural stem and progenitor cells is not fully understood, and insight into the mechanisms underlying its connection with cell fate regulation are lacking. The E2F3 transcription factor has emerged as an important regulator of neural precursor cell (NPC) proliferation in the embryonic and adult forebrain, and we demonstrate here that it also influences the self-renewal potential of NPCs. Using knockout mouse models of individual E2F3 isoforms, we demonstrate the surprising result that the classical transcriptional activator E2F3a represses NPC self-renewal and promotes neuronal differentiation, while E2F3b promotes the expansion of the NPC pool and inhibits differentiation. We attribute these opposing activities to a unique mechanism of transcriptional regulation at the *Sox2* locus, a key regulator of stem cell pluripotency, whereby E2F3a recruits transcriptional repressors to this site, and E2F3b promotes *Sox2* activation. Importantly, E2F3a-mediated *Sox2* regulation is necessary for cognitive function in the adult. Additionally, through the determination of genome-wide promoter binding sites for E2f3 isoforms as well as E2F4, another key regulator of NPC self-renewal, we determined that E2Fs are poised to regulate an extensive set of target genes with key roles in regulating diverse cell fate choices in NPCs, including self-renewal, cell death, progenitor expansion, maintenance of the precursor state, and differentiation. Together, these results reveal a diversity of function for E2Fs in the control of neural precursor cell fate, and identify E2F3 isoforms as important regulators of the pluripotency and stem cell maintenance gene *Sox2*.

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bHLH	basic helix-loop-helix
Bmi1	B-lymphoma Mo-MLV insertion region 1 protein
BMP	bone morphogenetic protein
Brca1/2	breast cancer 1/2
BrdU	Bromodeoxyuridine
C. elegans	*Caenorhabditis elegans*
Ccne	encoding cyclin E
Cdc	cell division cycle
Cdk	cyclin dependant kinase
CDKI	cyclin dependant kinase inhibitor
ChIP	chromation immunoprecipitation
Chip	microarray chip
CIHR	Canadian Institutes of Health Research
Cip/Kip	Cdk interacting protein/ kinase inhibitory protein
CNS	central nervous system
CP	cortical plate
Cre	cyclization recombination enzyme
CTCF	CCCTC binding factor
C-terminal	carboxy
Da	Dalton
DAVID	Database for analyzation, visualization and integrated discovery
Dlx	distaless related homologue
DNA	deoxyribonucleic acid
DP	DP transcription factor
E	Embryonic Day
EGF	Epidermal growth factor
E2F	E2 promoter binding factor (indicates the human protein)
E2f	E2 promoter binding factor (indicates the mouse protein)
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol tetra-acetic acid
EMSA	electrophoretic mobility shift assay
Ezh2	enhancer of zeste
FGF	Fibroblast growth factor
Fig	figure
flox	flanked loxP sites
Foxg1	forkhead box G1
FVB/N	Friend virus B type susceptibility/ NIH mouse
g	gram
GE	ganglionic eminence
G_0	gap 0 (quiescence)

G_1	gap 1 (interphase)
G_2	gap 2 (interphase)
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GO	Gene Ontology
GREAT	Genomic Regions Enrichment of Annotations Tool
H3K4Me3	Histone 3 tri-methylated at lysine 4
H3K27Me3	Histone 3 tri-methylated at lysine 27
HDAC	histine deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hes	hairy and enhancer of split homolog
Ink4	inhibitors of cdk4
IP	immunoprecipitation
IZ	intermediate zone
KO	knock-out
Lhx2	LIM homeobox gene 6
LxCxE	lysine- any amino acid- Cysteine- any amino acid- glutamic acid
M phase	mitosis
MB	marked box domain
MB	myoblast
MEF	mouse embryonic fibroblast
mRNA	messenger RNA
myc	myelocytomatosis viral oncogene
MZ	marginal zone
NES	nuclear export sequence
Ngn	neurogenin
Nkx2.1	Nk2 homeobox 1
NLS	nuclear localizarion sequence
NP	neural precursor
NP-40	Nonidet P-40
NPC	neural precursor cell
NSC	neural stem cell
N-terminal	amino
OGS	Ontario Graduate Scholarship
p	Probability value
Pax6	Paired box gene 6
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	potential of hydrogen
PNS	peripheral nervous system

pRB	retinoblatoma protein (indicates the human version)
pRb	retinoblatoma protein (indicates the mouse version)
RNA	ribonucleic acid
RT	room temperature
S phase	DNA synthesis
SAC	starburst amacrine
SEM	standard error of the mean
Shh	Sonic Hedgehog
shRNA	short hairpin RNA
Skp2	S-phase kinase-associated protein 2, the F-box protein of the SCF complex
Sox2	SRY (sex determining region Y)-box 2
Suv39h1	suppressor of variegation 3-9 homolog 1 (Drosophila)
SV40	Simian Virus 40
SVZ	subventricular zone
SWI/SNF	switching/sucrose non-fermenting
Tbr1	T brain 1
Tgf-b	transforming growth factor b
TSS	transcriptional start site
VZ	ventricular zone

CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview of pRB-E2F Pathway Function

The E2F and Retinoblastoma protein (pRB) families are best understood as fundamental cell cycle regulators, and constitute a pervasive tumour suppressive pathway. In the canonical model of pRB-E2F function, E2Fs bind to the promoters and activate expression of genes required for entry into the DNA synthesis phase (S phase) of the cell cycle, while pRB binds E2Fs to repress this activity primarily during gap 1 phase (G1) and in quiescent cells (Weinberg, 1995; Harbour, 2000). Research over the past decade has revealed that the true functional nature of this pathway is not nearly so simple. A diversity of cellular functions have now been described for the pRB-E2F pathway in addition to S phase entry; these include regulation of the DNA damage response, progression into mitosis, cellular differentiation, apoptosis, chromatin organization, processes related to embryonic and tissue development, and lipid metabolism (McClellan and Slack, 2007; Chen et al., 2009b). Multiple genes corresponding to each of these categories have been identified as direct E2F targets (Xu et al., 2007; Asp et al., 2009; Lee et al., 2011), demonstrating a much more expansive functional role for E2F transcriptional activity than was originally anticipated. Diverse roles for cell cycle regulatory proteins in the brain, outside of basic cell cycle regulation, have also been demonstrated. In parallel to this functional diversity, the mammalian pRB family consists of three unique members, while eight E2F genes have been identified, with two family members giving rise to two distinct protein isoforms. The degree of functional specificity and redundancy among E2F family members, as well as the extent of E2F regulated functions, are heavily contested questions. Broadly, this thesis will focus on an examination of these important questions, specifically regarding the nature of E2F function in neural stem and progenitor cells in the forebrain.

1.1.1 Identification of the pRB Tumour Suppressor

Historically, the Retinoblastoma gene (*RB1*) was originally identified as a tumour suppressor based on an association of its genetic locus with the development of an inherited form of eye tumour in children, termed Retinoblastoma (Francke and Kung, 1976; Yunis and Ramsay, 1978; Friend et al., 1986; Fung et al., 1987). Mutations in this locus were also linked early on to the development of osteosarcomas (Friend et al., 1986; Fung et al., 1987). *RB1* was infact the first tumour suppressor gene cloned (Lee et al., 1987a), and *RB1* mutations were subsequently linked to the development of additional forms of cancer, including small cell and non-small cell lung carcinoma, leukemia, and bladder, breast, cervical and prostate cancer (Harbour et al., 1988; Lee et al., 1988; T'Ang et al., 1988; Dyson et al., 1989; Bookstein et al., 1990; Horowitz et al., 1990; Hausen, 1991; Weinberg, 1991; Benedict, 1992; Weide et al., 1993). Further establishing pRB as an important tumour suppressor protein, it was found that certain DNA tumour viruses bind to pRB, inactivating its ability to repress cell proliferation, and that this interaction is important for viral oncogenic transformation (Whyte et al., 1989; Kaye, 2002; Münger, 2002). Furthermore, it was soon discovered that mutations in upstream regulators of pRB were also prevalent in a variety of tumour types, and it became clear that disruption of the pRB growth regulatory pathway may be a fundamental event for tumourigenesis in general (Sherr, 1996; Hanahan and Weinberg, 2000; Nevins, 2001).

The first insights into the molecular function of pRB were that it was predominantly localized to the nucleus, existed in hyper- and hypo-phosphorylated forms, was able to bind DNA, and was expressed in a wide range of tissues (Lee et al., 1987b; Weinberg, 1991). The *RB1* gene is very strongly conserved in evolutionary terms, with sequence homologues

and/or pRB expression having been widely observed in vertebrate, invertebrate and plant species (Lee et al., 1987b; Bernards et al., 1989; Destrée et al., 1992; Du et al., 1996; Cross and Roberts, 2001; Rotchell et al., 2001; Fay, 2002; Shen, 2002; Sabelli et al., 2005). In fact, examination of expression patterns in a panel of mouse tissues revealed that pRB is expressed ubiquitously (Bernards et al., 1989; Weinberg, 1991). These discoveries positioned pRB as a fundamental regulator of cell cycle control in virtually all cell types, and lead to an intense focus on deciphering the mechanisms by which pRB and its upstream and downstream regulators control the transition through the G1/S phase check-point. The majority of what we currently know about pRB function and its molecular interactions is derived from such cancer focused studies, aimed specifically at determining the mechanisms driving pRB-dependent cell cycle control.

1.1.2 Mechanistic Basis of Cell Cycle Regulation by pRB

The most well characterized function of pRB is its suppression of cell cycle progression, which is tightly linked with its ability to regulate multiple genes required for S-phase entry, the majority of which are controlled by E2F transcription factors (reviewed in (Knudsen and Knudsen, 2008)). In the basic cell cycle regulatory model (Figure 1.1), pRB is hypo-phosphorylated and bound to E2Fs during the G1 or G0 (quiescence) cell cycle phases, physically repressing the ability of E2Fs to promote gene expression by binding directly to their transactivation domain. Gene repression is achieved through the direct recruitment by pRB of chromatin remodeling proteins that can modify chromatin structure to repress transcription, or by interfering with assembly of the pre-initiation complex at E2F target promoters (Ross et al., 1999; Sutcliffe et al., 2000; Wirt and Sage, 2010). Chromatin

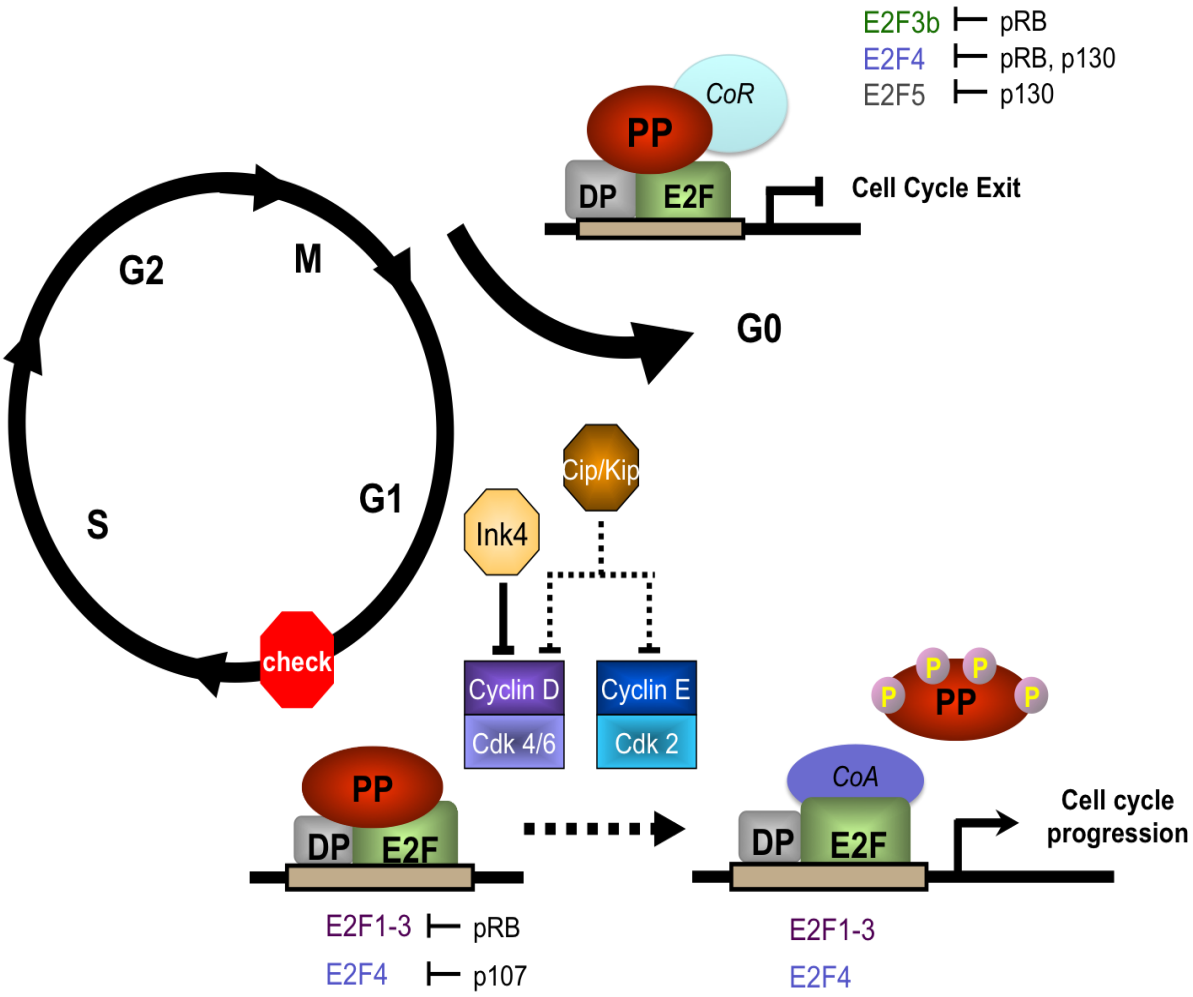


FIGURE 1.1 Model of pRB-E2F Molecular Function

E2F proteins heterodimerize with DP and interact at the promoter regions of target genes through a consensus DNA binding site. During G1 phase Pocket Proteins (PP) are bound to E2F at DNA sites, preventing their ability to activate transcription. As G1 progresses, Pocket Proteins are phosphorylated by Cyclin/Cdk complexes, and in their hyper-phosphorylated state are released from E2Fs, allowing entry into and progression through S phase. Pocket Proteins are maintained in their hyper-phosphorylated state in G1 due to Cyclin/Cdk inhibition by Cyclin-dependent kinase inhibitors (Ink4 and Cip/Kip). During S phase, E2Fs activate transcription by recruiting co-activators (CoA), such as histone acetyl-transferases. In G0 (a post-mitotic state), cell cycle exit is established by formation of active repression complexes between E2Fs and hyper-phosphorylated Pocket Proteins. Pocket Proteins recruit co-repressors (CoR) to E2F sites, which include histone-deacetylases, hostone methyl-transferases, and DNA methyl-transferases.

The most abundant E2F-Pocket Protein complexes in each cell cycle phase are shown. E2F1-4 are highly expressed during S phase, and these are repressed by pRB (E2F1-3) and p107 (E2F4) in G1 to prevent S phase entry. Alternatively, in G0 E2F3b-pRB, E2F4-pRB, E2F4-p130 and E2F5-p130 complexes are found. Modified from McClellan & Slack, 2007

remodeling proteins bound by pRB include histone deacetylases (such as HDAC1), histone methyl-transferases (such as Suv39h1/2), DNA methyl-transferases (DNMT1/2), and the ATP-dependent SWI/SNF modifiers (Strober et al., 1996; Brehm et al., 1998; Ferreira et al., 1998; Magnaghi-Jaulin et al., 1998; Robertson et al., 2000; Ferreira et al., 2001; Vandel et al., 2001; Pradhan and Kim, 2002; Vaute et al., 2002; Yoshimoto et al., 2006; Dick, 2007). The evidence to date indicates that this association of pRB with chromatin modifying proteins may be more relevant in the establishment of permanent gene repression associated with cell cycle exit into the G0 phase, as opposed to transcriptional regulation in cycling cells, as mutations within pRB's LXCXE domain through which chromatin associated proteins interact do not impair the ability of pRB to repress cell division (Isaac et al., 2006; Dick and Rubin, 2013). Furthermore, an important role for LXCXE-dependent interactions has been demonstrated in the context of cellular senescence and regulation of pericentric heterochromatin relevant to genomic stability and the progression through mitosis (Isaac et al., 2006; Talluri et al., 2010). A recent study has, however, challenged the viewpoint that pRB controls the post-mitotic state in all circumstances through LXCXE interactions, by demonstrating that disruption of the LXCXE domain in post-mitotic cortical neurons does not result in their re-entry into the cell cycle (Andrusiak et al., 2013). Thus, the requirement for pRB-mediated chromatin regulation in the context of cell cycle repression is still under active investigation.

pRB becomes increasingly phosphorylated throughout G1, coincident with the appearance of mitogenic stimuli that promote the expression of D type Cyclins, first due to Cyclin D in complex with CDK4 or CDK6 and then later in G1 by CDK2 associated with Cyclin E or Cyclin A (Harbour et al., 1988; Hinds et al., 1992; Knudsen and Wang, 1997;

Lundberg and Weinberg, 1998). By late G1 pRB exists in a hyper-phosphorylated state, which causes its release from DNA-bound E2Fs and thereby allows E2Fs to transcribe genes required for S phase entry, resulting in progression through the cell cycle (Figure 1.1). Throughout mitosis, pRB is de-phosphorylated by phosphatases and returned to its 'active' hypo-phosphorylated state for the next G1 phase (Tamrakar et al., 2000; Vietri et al., 2006). Anti-mitogenic signals, such as those initiated following DNA damage, can also return pRB to its hypo-phosphorylated state; these act by inhibiting Cyclin expression, inducing expression of CDK inhibitors, or by promoting pRB-directed phosphatase activity (Mittnacht, 1998; Knudsen et al., 2000; Avni et al., 2003). Hypo-phosphorylation of pRB is maintained in early G1 largely due to the presence of the CDK inhibitor proteins p16-INK4A, p21-CIP1 and p27-KIP1 (Serrano et al., 1993; Kamb et al., 1994; Sherr and Roberts, 1999; Liu et al., 2007; Knudsen and Knudsen, 2008). Reflecting this mechanistic model and a close functional association between pRB and E2F, pRB-E2F complexes are typically most abundant during cellular quiescence, and "free" E2Fs, unbound by pRB, are abundant in actively proliferating cells (Dyson, 1998). Furthermore, over-expression of E2F will force quiescent cells back into a cycling state even in the absence of growth factor stimulation (Johnson et al., 1993), while over-expression of pRB in certain cell lines leads to a cell cycle arrest that can be overcome by concomitant over-expression of E2Fs (Qin et al., 1995; Lukas et al., 1996; Mulligan and Jacks, 1998).

1.1.3 Comparative Roles of Pocket Protein Family Members

The Retinoblastoma protein is a 110 kDa phosphoprotein that belongs to a family of three structurally and functionally related proteins. The 'pocket protein' family includes pRB as

well as p107 and p130, and is so named because all three proteins share a structural domain, termed the 'pocket domain', through which they form contacts with many cellular proteins, including viral oncoproteins, chromatin remodeling proteins, and E2Fs (Hu et al., 1990; Wirt and Sage, 2010; Dick and Rubin, 2013). As with pRB, p107 and p130 were originally identified through their interaction with the DNA tumour viruses SV40 Large T antigen and E1A (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993). Early evidence into the relative functions of pocket protein family members revealed that, together, they control proliferation in most, and potentially all cell types (Cobrinik, 1996; Nevins, 1998; Cobrinik, 2005). Overall, p107 and p130 share approximately 54% sequence identity with each other and 25% identity with pRB, with the highest degree of sequence identity falling within the pocket domain (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993; Lin et al., 1996; Mulligan and Jacks, 1998). Most unicellular and lower organisms, including yeast, *Caenorhabditis elegans*, and the unicellular alga *Chlamydomonas reinhardtii*, have only one *Rb*-related gene, while *Drosophila*, which is higher up on the evolutionary scale, has two related family members (*RBF1* and *RBF2*) (reviewed in (Wirt and Sage, 2010)). All three pocket proteins function to control cellular proliferation, bind and regulate E2fs and are inactivated by Cyclin-CDK complexes (Chellappan et al., 1991; Cao et al., 1992; Fattaey et al., 1993; Zhu et al., 1993; 1993; Classon, 2001; Stengel et al., 2009). Sequence analysis also suggests that Cyclin-CDK dependent phosphorylation should result in similar structural changes among all three family members (reviewed in (Dick and Rubin, 2013)). Interestingly, while all three pocket proteins inhibit cell cycle progression and repress E2F-dependent transcription, only pRB is commonly associated with tumourigenesis (Knudsen and Knudsen, 2008), suggesting that

pocket protein functions are not completely redundant. Additionally, examination of the sequence of *Rb*-related genes across species reveals that *Rbl1* (p107) and *Rbl2* (p130) more closely resemble the "ancestral" *Rb* gene than does *Rb1* itself, suggesting that the tumour suppressive function of pRB may be a more recent evolutionary adaptation.

Early evidence supporting non-redundant functions for pocket proteins were the findings that pRB, p107 and p130 exhibit specific differences in their cell cycle dependent expression patterns and the E2Fs that they interact with. Interestingly, pRB is expressed in both proliferating and quiescent cells, while p130 is predominant in cells that have exited the cell cycle, and p107 levels are typically high during proliferation but low in quiescent populations (Beijersbergen et al., 1995; Kiess et al., 1995; Mayol et al., 1995; 1996; Hurford et al., 1997; Garriga et al., 1998; Smith et al., 1998; Burkhart et al., 2008).

In quiescent and senescent cells, where a stable cell cycle arrest is in place, p130 is the most abundant pocket protein family member (Moberg et al., 1996; Hurford et al., 1997), where it represses genes that function to promote cell cycle re-entry largely in collaboration with E2F4 and to a lesser extent with E2F5 (Figure 1.1) (Vairo et al., 1995; Smith et al., 1996; Lindeman et al., 1997; Trimarchi and Lees, 2002; Attwooll et al., 2004; Balciunaite et al., 2005; Litovchick et al., 2007). p107 also interacts with E2F4 and E2F5, but primarily at target gene promoters in cycling cells, and is detected in both the nucleus and cytoplasm of many cell types (Dyson et al., 1993; Ginsberg et al., 1994; Moberg et al., 1996; Lindeman et al., 1997; Wirt and Sage, 2010). Contrary to other pocket proteins, p107 expression levels and the abundance of p107-E2F4 complexes are highest in S phase (Zini et al., 2001); thus, it is thought that p107 binds to repressor E2Fs both within and outside of the nucleus to recruit them to target DNA sites as they are being actively transcribed (Verona et al., 1997; Puri et

al., 1998; Wirt and Sage, 2010). Alternatively, pRB binds preferentially to the growth promoting E2Fs, E2F1-3, to repress their transactivation potential, as well as E2F4 (Moberg et al., 1996; Chen et al., 2002; Lee et al., 2002; Trimarchi and Lees, 2002). While the contributions of individual E2Fs can differ depending on the tissue type, the association of pRB with E2F1-3 is important in facilitating and maintaining cell cycle arrest as well as cell survival *in vivo* (Saavedra et al., 2002; Chong et al., 2009b).

1.2 Overview of the E2F Protein Family

The E2F transcription factor was first defined as a cellular transcription factor capable of binding to the adenovirus E1A inducible E2 promoter ('E2F' = **E2 F**actor) and promoting its expression (Kovesdi et al., 1986; 1987). Subsequently, E2F became one of the first binding partners of the Retinoblastoma protein to be discovered, which sparked tremendous interest into the potential role of E2F in regulating the cell cycle (Bagchi et al., 1991; Chellappan et al., 1991; Chittenden et al., 1991; Helin et al., 1992; Kaelin et al., 1992; Bandara et al., 1993). Numerous transcription factor binding partners of pRB have been identified (Morris and Dyson, 2001), including additional transcription factors and a number of chromatin-associated proteins (Morris and Dyson, 2001; Goodrich, 2006), but its regulatory interaction with E2F is paramount to its ability to regulate cell division, as loss of E2f 'activators' from *Rb1* deficient embryos suppresses proliferative phenotypes associated with pRB loss (Tsai et al., 1998; Ziebold et al., 2001; Saenz-Robles et al., 2007; Chong et al., 2009b; Wenzel et al., 2011). Thus, deciphering the mechanisms by which pRB controls E2F activity, and determining the functions regulated by E2F became a strong focus in the quest to understand the role of pRB in cancer.

Soon after its initial identification, it was discovered that E2F functions to promote cell proliferation by trans-activating the expression of genes required for the progression into and through S phase, and that pRB inhibits this activity through direct binding to E2F (Helin et al., 1993a). It was also discovered that E2Fs heterodimerize with one of two Differentiation-related Polypeptide proteins, DP1 and DP2, most commonly DP1, and that this binding interaction is required for stable binding of E2F to its DNA sites and to achieve its full transactivation potential (Bandara et al., 1993; Girling et al., 1993; Helin et al., 1993b; Wu et al., 1995; Shan et al., 1996). The role of E2Fs in promoting S phase entry was quickly linked to their ability to transcriptionally activate multiple genes required for DNA replication and to ensure continued cell cycle progression, and importantly to this effect, it was observed that E2F expression patterns varied throughout the cell cycle with peak expression coinciding with the G1 phase (Hsiao et al., 1994; Neuman et al., 1994; Johnson et al., 1994b; Sears et al., 1997; Leone et al., 1998; He et al., 2000; Leone et al., 2000). Some of the earliest identified E2F target genes include those encoding DNA Polymerase α, Cdc6, Orc1, Mini Chromosome Maintenance proteins (MCM2-7), Thymidine kinase (TK1), DHFR, PCNA, Cyclin A, Cyclin E, pRB, p107 and even E2F1 itself (Hurford et al., 1997; Leone et al., 1998; Takahashi et al., 2000; Wells et al., 2000). The list of confirmed and putative E2F target genes has now expanded tremendously, as has the functional implications of these targets, and will be discussed further in later sections.

1.2.1 Comparative Roles of Activator and Repressor E2Fs

To date, eight *E2F* genes, giving rise to ten E2F proteins, have been identified in mammals (Chen et al., 2009c). E2F family members are largely defined based on sequence

conservation of the DNA binding domain, which imparts a common DNA binding specificity (Figure 1.2) (Kovesdi et al., 1987; La Thangue and Rigby, 1987; Ivey-Hoyle et al., 1993; Lees et al., 1993; Ginsberg et al., 1994; Li et al., 1994; Cartwright et al., 1998; Trimarchi et al., 2001; de Bruin et al., 2003a; Christensen et al., 2005; Logan et al., 2005; Maiti et al., 2005). An intense focus on the cellular roles of each family member following their discovery allowed for the division of E2Fs into three distinct classes. These classes include 'activator' E2Fs, which function primarily to promote the expression of E2F target genes, and two groups of 'repressor' E2Fs, either with or without the ability to interact with pocket proteins, whose primary role is to repress E2F target gene expression. The differential activities of activator and repressor E2fs are influenced by a variety of factors; these include unique cell cycle dependent expression patterns, differential degradation throughout the cell cycle, distinct subcellular localization, and differences in the pocket protein family members that they bind. Thus, differential regulation of both gene expression and protein interactions are thought to heavily underlie the unique transcriptional roles of the activator and repressor E2F sub-groups. In the next two sections I will overview the classical roles for activator and repressor E2Fs, defined from early studies based on cell culture models, and will discuss more recent data suggesting complexities in their functions in later sections.

1.2.1.1 Activator E2Fs

E2F1-3 comprise the 'activator' class of E2Fs, which is characterized by the ability of these family members to potently activate target genes and to drive S phase entry (Figure 1.2). These E2Fs are maximally expressed in late G1 to early S phase, which correlates precisely with their ability to promote entry into and progression through S phase

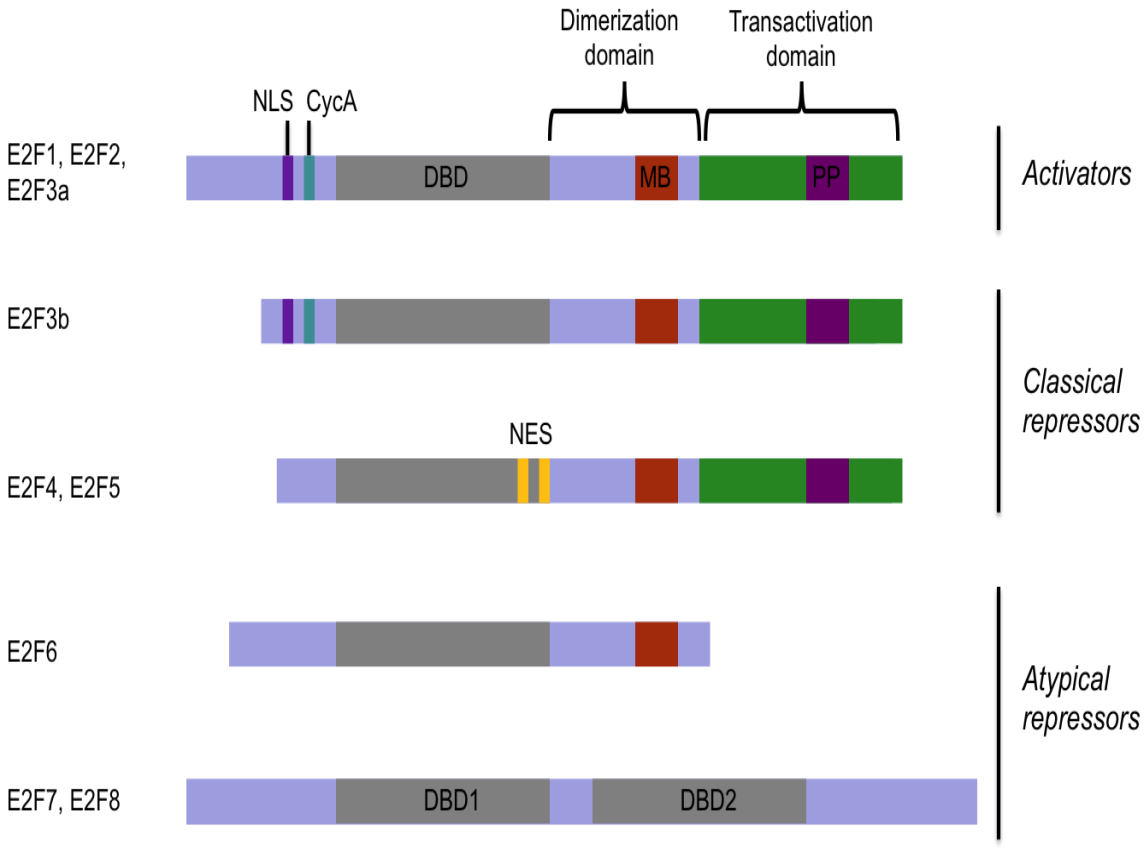


FIGURE 1.2 Structural Schematic of E2F Family Members

Structurally defined domains of E2F family members are demonstrated. Note that the classical activators (E2F1, E2F2, E2F3a) and E2F3b uniquely posses a nuclear localization sequence and a Cyclin A binding domain, while only E2F4 and E2F5 contain a pair of nuclear export sequences. E2F1-5 posses transactivation and Pocket Protein binding (PP) domains, while E2F6-E2F8 do not interact with pocket proteins. E2F6 does posses the DP dimerization domain. Note the conserved DNA binding domain in all E2F proteins. NLS = nuclear localization sequence; CycA = Cyclin A binding domain; MB = marked box domain; PP = pocket protein binding domain; NES = nuclear export sequence; DBD = DNA binding domain.

E2F family members were classified as ‘Activators’ or ‘Repressors’ based predominantly on their cell cycle-dependent expression patterns, as well as structural features. ‘Atypical repressors’ are so named due to their lack of Transactivation or Pocket Protein binding domains. Note that although not pictured here, two E2F7 proteins exist due to alternative splicing of the *E2f7* transcript.

(Hsiao et al., 1994; Neuman et al., 1994; Johnson et al., 1994b; Sears et al., 1997; Leone et al., 1998; 2000). In fact, early studies suggested that the growth promoting activator E2Fs are absolutely required for cellular proliferation. Studies of *dE2f* knockouts in drosophila demonstrated a lack of BrdU incorporation in the absence of the activator *dE2f1* (Duronio et al., 1995; Royzman et al., 1997), and E2f1-3 deficiency in cultured mouse fibroblasts results in cell cycle arrest (Wu et al., 2001). Furthermore, overexpression of E2F1, 2 or 3 is sufficient to drive serum starved fibroblasts into S phase (Johnson et al., 1993; Lukas et al., 1996), participate in the immortalization of primary keratinocytes (Melillo et al., 1994), and transform immortalized fibroblasts (Johnson et al., 1994a; Xu et al., 1995). It should be noted that more recent *in vivo* studies of activator E2F proteins in mouse models have revealed that their role in regulating cellular proliferation is more complex than these earlier studies suggested. These findings will be further discussed below. Structurally, the activator E2Fs are differentiated from repressors by their longer N-terminus. The function of the extended N-terminal sequences is not fully understood, however structural domains within this region have shown to be important for specific aspects of E2F regulation and function, predominantly related to their cell cycle dependent expression patterns. First, E2F1-3 contain a conserved domain within this region through which Cyclin A has been shown to bind E2F1 and inhibit its ability to bind DNA during the S and G2 cell cycle phases (Krek et al., 1994; 1995; Dynlacht et al., 1997). E2F1 has also been shown to be targeted for degradation during S/G2 via an N-terminal interaction with the ubiquitin-protein ligase protein SCF-SKP2 (Marti et al., 1999). Finally, the E2F1 N-terminus has also been linked to inhibition of the elongation phase of DNA replication during S phase (Stubbs and Hall, 2002). As discussed in section 1.1.3, E2F1-3 primarily associate with pRB, which serves to repress transcriptional

activation until the onset of S phase and to form repressive complexes during differentiation (Chong et al., 2009b; Chen et al., 2009c).

1.2.1.2 Repressor E2Fs

The 'repressor' class of E2Fs includes E2F4-8 and may include E2F3b, although this family member has been difficult to characterize in such a precise, categorical manner (will be discussed in section 1.2.3) (Figure 1.2). Repressor E2Fs function primarily in quiescent and post-mitotic cells to establish and maintain a non-proliferative state (Wu et al., 2001; Trimarchi and Lees, 2002; Chen et al., 2009c). This group of E2Fs can be further sub-divided into 'typical' and 'atypical' repressors. Typical repressors (including E2F4 and E2F5) interact with pocket proteins, predominantly p107 and p130 (Dyson, 1998; Cobrinik, 2005; Chen et al., 2009c), and are expressed throughout the cell cycle in proliferating and quiescent cells, contrary to the cell cycle dependent expression pattern of activator E2Fs (Sardet et al., 1995; Moberg et al., 1996; He et al., 2000; Leone et al., 2000). E2F4 is also an important binding partner of pRB in target gene repression. Atypical repressors (including E2F6-8) lack the C-terminal pocket protein binding domain and are thus presumed to repress E2F target genes in a pocket protein-independent manner (Cartwright et al., 1998; Gaubatz et al., 1998; Di Stefano et al., 2003; Christensen et al., 2005). While E2F6 has retained the ability to dimerize with a DP protein, E2F7 and E2F8 don't bind DP and instead form homo- and hetero-dimers with one another (Cartwright et al., 1998; Di Stefano et al., 2003; LI et al., 2008). Additionally, atypical repressors demonstrate unique expression patterns, where E2F6 is induced at the G0-G1 transition (Trimarchi et al., 1998) and E2F7 and E2F8 levels peak during the S and G2 phases (LI et al., 2008). The co-repressors with which atypical E2Fs

associate to control gene expression are largely unknown, however E2F6 has intriguingly been identified as a member of Polycomb protein complexes (Trimarchi et al., 2001). Pocket protein-E2F repressive complexes are thought to play a role in establishing and maintaining a differentiated or quiescent state, as they function to recruit chromatin remodeling complexes to the promoters of target genes to actively repress transcription from the promoter (Dyson, 1998; Dick and Rubin, 2013). Consistent with a repressive role for E2F4 and E2F5, their overexpression *in vivo* or in serum starved fibroblasts does not drive cells into S phase (Lukas et al., 1996; Muller et al., 1997; Verona et al., 1997; Chen et al., 2004b), contrary to the function of E2F1-3. This fundamental functional difference between activator and 'typical' repressor E2Fs is thought to largely be due to their differential sub-cellular localization: while E2F1-3 are predominantly nuclear due to their nuclear localization sequence (NLS) (Magae et al., 1996; Muller et al., 1997; Verona et al., 1997), E2F4 and E2F5 are most abundant in the cytoplasm and contain a nuclear export sequences (NES) (Gaubatz et al., 2001). E2F4 and E2F5 are recruited to the nucleus in G0 and G1 phases via their interaction with pocket proteins, in order to repress E2F target gene expression (Verona et al., 1997; Trimarchi and Lees, 2002).

1.2.2 An Essential Role for E2F3 in Proliferation and Embryonic Development

Functional comparisons of E2F family members via single and compound *E2f* knockout mouse models have revealed that among the activator E2F class, the *E2f3* gene plays the most critical role in mouse development and regulation of proliferation (Wu et al., 2001; Tsai et al., 2008). While mice deficient in E2f1, E2f2 or a combination of both (*E2f1-/-; E2f2-/-*) are viable and developed to adulthood, *E2f1-/-;E2f3-/-* and *E2f2-/-;E2f3-/-* mice died by day

E9.5 of embryogenesis, and single *E2f3* deficient animals on a pure background (FVB, C57BL/6J or 129/Sv) die around E12.5 (Humbert et al., 2000b; Wu et al., 2001; Cloud et al., 2002; Tsai et al., 2008), as discussed above. Furthermore, fibroblasts deficient for both *E2f1* and *E2f2* show no proliferative defect, while concomitant loss of *E2f3* in cells deficient for *E2f1*, *E2f2* or both, or loss of *E2f3* alone, results in severe impairment in proliferative potential, accompanied by a lack of expression of a number of cell cycle related E2f target genes (Leone et al., 1998; Wu et al., 2001). E2F3 has also emerged as a critical binding partner of pRB in controlling cell survival and proliferation during development and tumourigenesis. For instance, loss of *E2f3* rescues the ectopic proliferation and apoptosis observed in the developing lens, CNS and PNS of *Rb1-/-* embryos (Ziebold et al., 2001; Saavedra et al., 2002). Additionally, *E2f3* deficiency in *Rb1+/-* mice dramatically alters their tumour spectrum, such that the development of pituitary tumours characteristic of pRb+/- mice is suppressed, but development of metastatic thyroid carcinomas is dramatically increased, due to an increase in the rate of tumour initiation (Ziebold et al., 2003). This suggested that E2F3 may have the ability to function as both a transcriptional activator and repressor, in a context specific manner. Indeed, a recent study demonstrated that in the context of tissue development E2F1-3 function as activators in proliferating cells and as pRB-associated repressors in differentiating cells (Chong et al., 2009b); as E2F3 is the most abundant E2F protein present in pRB complexes (Leone et al., 2000), this observation holds particular relevance for E2F3 function.

1.2.3 Two Isoforms are Expressed From the *E2F3* Locus: E2F3a and E2F3b

The findings discussed above demonstrated an essential role for E2F3 in embryonic development, cellular proliferation and survival as both a transcriptional activator and a repressor. *E2F3* was originally considered to be a single protein coding gene, like its fellow family members, however we now know that two protein isoforms are transcribed from the *E2F3* locus through the use of alternative promoters (Adams et al., 2000; He et al., 2000; Leone et al., 2000). These two isoforms, termed E2F3a and E2F3b, share identical C-terminal sequences which encompass the bulk of the E2F3 sequence and encode the NLS, Cyclin A binding, DNA binding, DP dimerization, Marked Box and transactivation/ Pocket protein binding domains (He et al., 2000; Leone et al., 2000). The inclusion of the NLS and Cyclin A binding domains in sequences of both E2F3 isoforms is consistent with a common identity as activator E2Fs; however, their N-termini are quite distinct, with E2F3a and E2F3b possessing 121 and 6 unique N-terminal residues, respectively. E2F3b is in fact missing the Ubiquitin targeting domain found in E2F3a, suggesting that their expression may be regulated in a differential manner (Leone et al., 2000). As E2F3a contains all defined domains present in other activator family members, its structure suggests that E2F3a can account for the activator function previously observed for the *E2F3* gene product. E2F3b, however, is difficult to classify purely based on structural information, as its structure contains features of both the activator and repressor E2F classes.

Analysis of E2F3a and E2F3b expression patterns in quiescent and serum stimulated rat embryonic fibroblasts demonstrated that E2F3a expression is indeed cell cycle regulated, appearing in late G1 and peaking during S phase, mirroring the expression pattern of other activator E2Fs (Leone et al., 2000). Alternatively, E2F3b is expressed ubiquitously in

quiescent cells and throughout the cell cycle (He et al., 2000; Leone et al., 2000), a classical feature of repressor E2Fs. It was subsequently shown that expression of E2F3a is negatively regulated through a series of four E2F binding sites located between 150bp and 1415bp upstream of its transcriptional start site (TSS), again similar to the regulation of other activator E2Fs (Hsiao et al., 1994; Neuman et al., 1994; Johnson et al., 1994b; Sears et al., 1997), and that binding of the Myc transcription factor to a group of E-box elements in the E2F3a 5' region is required for full activation of E2F3a following serum stimulation (Adams et al., 2000). E2F3b, on the other hand, lacks these regulatory elements in its 5' region, and its expression is not induced by Myc (Adams et al., 2000). This expression data further solidified E2F3a as an activator E2F along with E2F1 and E2F2, and suggested that E2F3b functions as a repressor, akin to E2F4 and E2F5. Supporting this, E2F3b was indeed found to form repressive pocket protein complexes in quiescent cells. However, setting it apart from E2F4 and E2F5, which partner with p130 and to a lesser extent p107 in this context, E2f3b specifically bound to pRb and represented the most abundant E2f-pRb complex in quiescent cells (Leone et al., 2000). E2f3b was soon demonstrated to be required for repression of the *Arf* tumour suppressor locus in MEFs, implicating E2F3b-mediated repression as an important tumour surveillance mechanism (Aslanian et al., 2004). Thus, these early findings of the overlapping and unique features of E2F3 isoforms suggested that E2F3a likely functions as a classical activator E2F, and E2F3b as a repressor by forming pocket protein complexes with pRB. Given these observations and the fact that the E2F3a and E2F3b proteins are predominantly identical in sequence, it was suggested early on that they may functionally counter one another by activating and repressing overlapping sets of target genes (Adams et al., 2000; Leone et al., 2000).

1.3 Cell Cycle Independent Functions of the pRB-E2F Pathway

Early studies into the function of the pRB-E2F pathway offered invaluable insights into the basic mechanisms of cell cycle regulation and tumour suppression; however, more recent advances, based on knockout mouse models and large-scale evaluation of E2F target genes, have revealed an incredible diversity of function for pocket proteins and E2Fs that cannot be fully appreciated with the simple cell cycle regulatory model. They have also revealed both redundant and specific functions for E2F family members, broadening the complexity of E2F function.

1.3.1 A Requirement for pRB in Embryonic Development

The first hints that the pRB pathway plays an important functional role beyond cell cycle control and can exhibit tissue specific functions came from studies of *Rb1* knockout mice. Germline deficiency for *Rb1* resulted in embryonic lethality between E13.5 and E15.5, and knockout embryos demonstrated hematopoietic defects marked by an increased number of immature nucleated erythrocytes, as well as ectopic mitosis and extensive apoptosis throughout the developing nervous system, most predominantly within the hindbrain (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Subsequently, defects in lens development were observed in *Rb1* knockouts, marked by inappropriate S phase entry preceding cell death (Morgenbesser et al., 1994). Interestingly, unlike humans heterozygous for *RB1, Rb1*+/- mice do not develop retinoblastoma. Instead, they are predisposed to thyroid medullary carcinoma (Williams et al., 1994; Dick and Rubin, 2013) and adenocarcinoma of the intermediate lobe of the pituitary (Jacks et al., 1992; Hu et al., 1994; Maandag et al., 1994); these tumours typically demonstrate loss of the wild-type (WT) *Rb1* allele.

Subsequent analysis of *Rb1-/-* embryos that had WT placentas showed that the early embryonic lethality of the germline *Rb1* knockouts, as well as the massive central nervous system (CNS) apoptosis and the majority of the hematopoietic abnormalities, resulted from hypoxic stress due to abnormal placental development in the absence of pRb (MacPherson et al., 2003; Wu et al., 2003; de Bruin et al., 2003b; Wenzel et al., 2007). In fact, these mice die at birth due to severe skeletal muscle defects, marked by hypoplastic and dysplastic myofibers within the diaphragm, limbs, intercostal muscles and tongue. Additionally, although the CNS apoptosis observed in germline *Rb1* knockouts was rescued in these animals, ectopic proliferation throughout the CNS was still present, as was both the proliferative and apoptotic defects previously observed in the lens. These findings suggested an essential role for the pRB pathway in embryonic development and post-natal survival, and demonstrated a marked tissue specificity in the nature and extent of its function. Furthermore, given the apoptotic phenotypes observed, they also suggested potential roles beyond cell cycle control.

Contrary to pRb, mice deficient in either p107 or p130 are viable, fertile and display no overt phenotypes (Cobrinik et al., 1996; Lee et al., 1996). Analysis of compound knockout mice, however, has revealed extensive functional overlap between these two pocket proteins, as mice lacking both p107 and p130 exhibit perinatal lethality, displaying defects in chondrocyte and epidermal differentiation (Cobrinik et al., 1996; Ruiz et al., 2003). Mice lacking both pRb and either p107 or p130 are phenotypically similar to pRb knockouts but die earlier and show an exacerbation of proliferative and apoptotic phenotypes (Lee et al., 1996; Lipinski and Jacks, 1999; Sage, 2000; Berman et al., 2009); additionally, concomitant loss of p107 in *Rb1-/-* mice supplied with a WT placenta revealed novel

phenotypes including increased proliferation of blood vessel endothelial cells and heart defects (Berman et al., 2009). Finally, loss of all three pocket proteins results in lethality by E9.5-11.5 and increased proliferation and cell death in multiple cell types (Wirt et al., 2010). These findings therefore demonstrate a substantial degree of functional redundancy between pocket proteins, and highlight both cell cycle dependent and independent functions.

1.3.2 E2Fs Regulate Diverse Cellular Functions

Although original studies in cultured fibroblasts lacking activator E2Fs suggested that they are required for cellular proliferation (Wu et al., 2001), three recent studies using mouse models in which all three activator E2Fs are lacking have revealed the unexpected finding that absence of E2F1-3 does not lead to gross proliferative defects *in vivo* in this system. In fact, E2F1-3 are dispensable for normal proliferation *in vivo*, apparently in the majority of tissues, and instead appear to be communally required for cell survival (Chen et al., 2009a; Chong et al., 2009b; Wenzel et al., 2011). Similarly, the repressors E2F4 and E2F5 were also shown to be dispensable for the regulation of cell cycle progression *in vivo*; however they are required to establish a permanent cell cycle arrest *in vitro* (demonstrated in MEFs and immortalized 3T3 cells) (Gaubatz et al., 2000). Embryos lacking all three activator E2fs die between E9.5 and E11.5, with live embryos appearing morphologically normal and exhibiting no obvious proliferation defects in most tissues. Interestingly, E2Fs demonstrated a clear role in cell cycle regulation only in differentiating cells (shown specifically in the intestinal villi and lens epithelium), where they function as transcriptional repressors in collaboration with pRb to enforce a cell cycle arrest (Chong et al., 2009b; Wenzel et al., 2011). These studies demonstrated specifically that deficiency of activator E2Fs in retinal

progenitors (Chen et al., 2009a), lens epithelial progenitors (Wenzel et al., 2011), embryonic stem cells and intestinal stem and progenitor cells (Chong et al., 2009b) does not lead to proliferation defects but to defects in cell viability, due to both p53- dependent and independent mechanisms depending on the cell type. These findings lead to some very surprising conclusions: first, E2F1-3 are required to inhibit cell death *in vivo*, when activator E2Fs have been demonstrated otherwise to be pro-apoptotic; second, not only are E2Fs capable of cell cycle independent functions, but their primary cellular role *in vivo* appears to be independent of classical cell cycle control. A primary role for E2Fs outside of cell cycle regulation is further supported by findings in the nematode *Caenorhabditis elegans*, where the E2F orthologue *elf-1*, along with *dpl-1*, is essential for fertility by controlling differentiation related cell fate processes as opposed to proliferation (Ceol and Horvitz, 2001; Myers and Greenwald, 2005).

1.3.2.1 Knockout Mouse Models Reveal Specificity of E2F Function

Animal models of individual *E2f* knockouts have revealed diverse functional roles for E2Fs and have further challenged the canonical model of E2F function. For instance, *E2f1* knockout mice exhibit testicular atrophy, decreased thymocyte apoptosis and hyper-proliferation and tumourigenesis in various tissues, predominantly the thymus, lungs and reproductive tract (Field et al., 1996; Yamasaki et al., 1996). E2f1 deficient mice also exhibit impaired β-cell function and pancreatic growth (Fajas et al., 2004), and E2F1 has further been implicated in the regulation of cellular respiration and the switch between glycolytic and oxidative metabolism (Blanchet et al., 2011). These findings have challenged the original model of a strictly pro-proliferative role for E2F1 by demonstrating that it can also

function as a tumour suppressor by repressing proliferation and promoting apoptosis, as well as a regulator of diverse developmental processes. E2F2 also exhibits tumour suppressive features *in vivo*, marked by an accumulation of T lymphocytes leading to the development of autoimmune disease in *E2f2-/-* mice (Murga et al., 2001). Alternatively, E2F3 does not exhibit tumour suppressive function *in vivo* (Humbert et al., 2000b). In fact, loss of the *E2f3* locus results in a strikingly different phenotype compared to other E2f 'activator' knockouts, with *E2f3-/-* mice exhibiting embryonic lethality as early as E12.5 in pure background strains, reduced viability on a mixed background, and premature death due to cardiac defects in those that do survive postnatally (Humbert et al., 2000b; Cloud et al., 2002; Tsai et al., 2008). Analysis of individual E2f3 isoform knockouts revealed much subtler phenotypes than loss of the entire *E2f3* locus. These mice are viable and fertile, and E2f3a deficient animals exhibit a relatively mild phenotype whereby aged animals have a thinner appearance due to a reduction of white adipose tissue (WAT) (Danielian et al., 2008; Tsai et al., 2008); *E2f3b-/-* mice, on the other, display no overt phenotypes (Tsai et al., 2008).

Analysis of E2f 'repressor' knockouts has revealed even more divergent tissue specific functions for individual E2fs. *E2f4* knockouts die embryonically due to opportunistic infection resulting from craniofacial defects (Humbert et al., 2000a). They also exhibit defective differentiation of gut epithelium and multiple hematopoietic cell lineages, but show few obvious proliferative defects, aside from impaired erythroid proliferation that leads to macrocytic anaemia (Rempel et al., 2000; Humbert et al., 2000a; Kinross, 2006). E2f5 deficient mice develop hydrocephalus, resulting from excessive production and secretion of cerebral spinal fluid, and die perinatally (Lindeman et al., 1998). These mice also lack an obvious proliferative phenotype. *E2f6* knockouts suffer testicular abnormalities and homeotic

transformations of the axial skeleton, underscored by a unique interaction of E2f6 with Polycomb complexes, although their life span is normal (Trimarchi et al., 2001; Ogawa, 2002; Storre et al., 2002). Lastly, deficiency of either of the atypical family members, E2f7 and E2f8, show no overt phenotypes (LI et al., 2008).

1.3.2.2 Evidence for Functional Redundancy Among E2Fs

Mouse models in which multiple E2fs are concurrently knocked-out have revealed additional phenotypes that are not observed in single protein knockouts, suggesting that E2Fs function redundantly or that they can functionally compensate for one another in certain contexts. For example, *E2f1-/-; E2f2-/-* mice develop non-autoimmune insulin-dependent diabetes and exocrine pancreatic dysfunction with age, due to exocrine cell dysplasia, polyploidy and apoptosis resulting in pancreatic atrophy (Li et al., 2003; Iglesias et al., 2004). Notably, these phenotypes are largely distinct from those observed in single *E2f1* or *E2f2* knockouts. Additionally, whereas *E2f7* and *E2f8* knockout mice presented with no obvious phenotypes, combined loss of *E2f7* and *E2f8* results in lethality by E11.5 with wide-spread apoptosis throughout the embryo, vascular dilation and hemorrhage, and no proliferative defects (LI et al., 2008). Additional mouse models have revealed both common and unique roles for E2f3 isoforms in mammalian development. These models and their phenotypes will be more thoroughly described in the following section, however the finding that the sole presence of either E2f3a or E2f3b can support cellular proliferation *in vitro* as well as embryonic development *in vivo* (Tsai et al., 2008) suggests extensive functional redundancy between E2F3 isoforms.

Supporting findings from animal models, studies investigating E2F target genes in various tissue types have found largely overlapping binding patterns for different family members, both within a given cell line and between multiple cell types (Ren, 2002; Xu et al., 2007; Gokhman et al., 2012). Importantly, they have identified thousands of genes whose proximal promoter regions are bound by E2Fs, the majority of which are involved in functions unrelated to cell cycle progression. A comprehensive analysis of E2f target genes in distinct cell types will allow for a clearer understanding of the degree of functional overlap and specificity among E2F family members as well as their functional roles. To date, these studies have focused predominantly on identifying the binding sites of E2F1 and E2F4 in a number of immortalized and transformed cell lines. A surprising finding has emerged from this work, in that the canonical activator E2F1 and the repressor E2F4 exhibit largely overlapping binding sites in proliferating populations, which are predominantly located very close to transcriptional start sites (TSS) at which POLR2A and TAF1 are bound (Xu et al., 2007), identifying regions that are transcriptionally active or are poised for activation. Strikingly, at least one E2F protein was found at the majority of all POLR2A/TAF1 bound promoters, suggesting that a bound activator or repressor E2F may signal the presence of transcription initiation complexes (Xu et al., 2007).

Importantly, these broad scale ChIP-based screens have highlighted extensive redundancy in the putative target genes bound by different E2F proteins, for which unique phenotypes have been described *in vivo*. This challenges the hypothesis that functions specific to individual E2Fs are the result of differential promoter binding. Speaking to this notion, an elegant set of experiments in which the coding sequences for E2f3b or E2f1 were expressed from the E2f3a locus demonstrated that the specific requirement for E2f3a for

post-natal development is not due to a unique function of the E2f3a protein itself, but to the regulatory sequences that dictate its spatial and temporal expression patterns (Tsai et al., 2008). Remarkably, in these experiments expression of either E2f3b or E2f1 from the E2f3a locus was sufficient to suppress the phenotypic defects observed in *E2f3a-/-* or *E2f1-/-;E2f3a-/-* mice. This suggests then that differential expression of E2F family members, as opposed to differences in the target genes that they bind and regulate, underlies their observed unique functions. A subsequent study demonstrated, however, that E2F3a and E2F3b cannot functionally compensate for one another at the phenotypic or gene regulatory level in differentiating muscle cells (Asp et al., 2009), demonstrating that regulation of unique target genes by different E2Fs can underlie specificity of function in at least certain contexts. Thus, a more comprehensive understanding of the target genes bound and regulated by different E2F family members in diverse tissue types, especially in primary cells for which data is currently lacking, is required to fully understand the extent of E2F function and the degree of redundancy and specificity within the E2F family.

1.4 E2Fs Function as Both Activators and Repressors *In Vivo*

In addition to identifying E2F bound regions, ChIP-chip and ChIP-Seq studies have also allowed for a direct determination of the change in expression levels of putative E2F target genes following knock-down or over-expression of individual E2F proteins. These analyses have consistently revealed the surprising finding that a similar number of E2F bound target genes are up-regulated as they are down-regulated when levels of a particular E2F are altered, with a similar pattern observed for classical ‘activator’ and ‘repressor’ E2Fs, suggesting that E2Fs actually function as both activators and repressors in proliferating cells

(Xu et al., 2007; Asp et al., 2009; Lee et al., 2011). This finding strongly contradicts the canonical model of E2F function, but supports previous evidence that traditional activator E2Fs are capable of transcriptional repression, while repressor E2Fs can also function as activators.

For example, E2F1 has been linked to the repression *in vivo* of a panel of genes involved in oxidative metabolism (Blanchet et al., 2011), and can directly repress expression of the anti-apoptotic gene *Mcl1* to promote apoptosis (Croxton et al., 2002). The increased proliferation of T-lymphocytes in *E2f2-/-* mice is thought to result from the loss of E2f2-mediated repression of cell cycle genes in the lymph node (Murga et al., 2001). Additionally, E2F3a and E2F3b have both been linked to transcriptional activation and repression in different contexts; these findings will be further discussed in the following section. Finally, as previously noted, E2F1-3 were demonstrated to function primarily as transcriptional activators in proliferating cells and as repressors in complex with pRb in differentiating cells within the intestinal villi (Chong et al., 2009b), demonstrating that canonical activator E2Fs actually function as both activators and repressors on a broad scale.

While the traditional repressor E2f4 has been strongly linked to target gene repression to maintain a non-proliferative state (Litovchick et al., 2007; Dick and Rubin, 2013), a number of observations have been made that also suggest a pro-proliferative, transcriptional activation role for E2F4. For instance, loss of E2f4 in *Rb1* deficient mice rescues tumour development in *Rb1+/-* or *Rb-/-* chimeric mice, suggesting that E2f4 promotes the expression of genes required for cell cycle entry (Lee et al., 2002). Additionally, mutant alleles of *E2f4*, some known to result in a protein unable to bind and be repressed by pocket proteins or in increased transcript levels, have been identified in a

number of tumour types in humans, including gastrointestinal, breast, prostate, and hepatocellular carcinmoas (Souza et al., 1997; Schwemmle and Pfeifer, 2000)
Strikingly, overexpression of E2f4 in stratified epithelial tissue *in vivo* induces proliferation in the epidermis at rates comparable to those achieved following overexpression of E2f1, and E2f4 overexpressing mice develop tumours. Speaking again to unique functions for E2fs *in vivo*, mice overexpressing E2f1 do not develop tumours, this is attributed to induction of apoptosis by E2f1, which does not occur following E2f4 expression (Wang et al., 2000).

1.4.1 Relative Roles of E2F3 Isoforms *In Vivo*

In vitro based assays have demonstrated that both E2F3a and E2F3b can efficiently activate transcription from E2F responsive promoters in reporter assays, although E2F3b is somewhat less potent than E2F3a (He et al., 2000; Chong et al., 2009a). This suggests that both isoforms are actually capable of functioning as transcriptional activators, and indeed both isoforms are able to bind the promoters of E2F target genes following serum stimulation (Chong et al., 2009a), consistent with cell cycle regulated activator E2F activity.
Additionally, both *E2f3a-/-* and *E2f3b-/-* MEFs proliferate at a decreased rate and demonstrate a significant defect in their ability to re-enter the cell cycle from quiescence, and demonstrate reduced expression of cell cycle related E2F target genes (Danielian et al., 2008; Chong et al., 2009a). As described above, *E2f3a* and *E2f3b* deficient mice are viable, fertile, live a normal life-span, and display no externally obvious phenotypes aside from a thinner appearance due to decreased WAT in aged *E2f3a-/-* mice (Tsai et al., 2008).
Correspondingly, the presence of either E2f3a or E2f3b alone, in the absence of the remaining activator E2Fs (*E2f1-/-; E2f2-/-; E2f3a-/-* and *E2f1-/-; E2f2-/-; E2f3b-/-*), is

sufficient for cellular proliferation *in vitro* and to support embryonic development (Tsai et al., 2008; Chong et al., 2009a). As deficiency of both E2f3 isoforms leads to lethality in mid embryogenesis, and the S phase entry defect of *E2f3-/-* MEFs is much more severe than individual isoform knockouts(Leone et al., 1998; Cloud et al., 2002; Tsai et al., 2008), these findings demonstrate a substantial degree of functional redundancy between E2f3a and E2F3b in regulation of proliferation and embryonic development, and suggest that both function as transcriptional activators.

A few studies have initiated an investigation into the tissue-specific functions of E2F3 isoforms, and these have revealed that the relative functions of E2F3a and E2F3b *in vivo* can vary greatly depending on the context. Surprisingly, due to the high abundance of E2F3b-pRB complexes, E2f3a was found to account for a greater majority of phenotypes observed in *Rb1-/-* embryos than was E2f3b. Specifically, loss of E2f3a, but not E2f3b, in *Rb1-/-* mice rescued the ectopic proliferation and unscheduled apoptosis in the placenta, fourth ventricle of the hindbrain, and the dorsal root ganglia of the peripheral nervous system, while repression of these phenotypes in lens fiber cells is dependent on a combination of both E2f3a and E2f3b (Chong et al., 2009a). Strikingly, mice expressing only E2f3a (*E2f1-/-;E2f2-/-;E2f3b-/-*) lived well into adulthood, albeit with reduced body weight, while *E2f1-/-;E2f2-/-;E2f3a-/-* mice died perinatally, highlighting an important role for E2f3a specifically in post-natal development (Tsai et al., 2008). Post-natal development was further shown to depend on the presence of either E2f3a or E2f1, as *E2f1-/-;E2f3b-/-*, *E2f2-/-;E2f3a-/-*, or *E2f1-/-;E2f2-/-* mice were viable, fertile and lived into adult-hood with no overt phenotypes (aside from the pancreatic defect described above in *E2f1-/-;E2f2-/-* mice) while *E2f1-/-;E2f3a-/-* mice were severely impaired (Tsai et al., 2008). Although visibly normal at

birth, *E2f1-/-;E2f3a-/-* mice became severely runted by their third week of life, demonstrating a large reduction in the size and proliferative index of most body organs, and the majority of these animals died within their first month of life likely due to multi-organ failure (Danielian et al., 2008; Tsai et al., 2008). Most notably, these mice suffered from under-developed testes and ovaries, reduced WAT deposits, fewer pancreatic exocrine cells, and cartilage defects marked by disorganization and abnormal morphology of chondrocytes. These findings demonstrate an important role for E2F3 isoforms, but predominantly E2F3a, in proliferation and cell survival *in vivo*. Interestingly, a pair of studies has implicated both E2F3a and E2F3b in the promotion of differentiation, but under very different contexts. The differentiation of starburst amacrine cells (SACs), an interneuron sub-type within the retina, was shown to be dependent on pRb-mediated regulation of E2f3a (Chen et al., 2007), while E2f3b was shown to promote the differentiation of cultured myoblasts into myotubes independently of pRb (Asp et al., 2009). The latter study demonstrated the important result that while E2f3 isoforms bind a set of overlapping target genes in proliferating myoblasts, they also bind the promoters of a large number of unique target genes in both myoblasts and differentiated myobtubes (Asp et al., 2009). Furthermore, this analysis demonstrated that while cell cycle regulatory genes are targeted primarily by E2f3a in myoblasts, genes related to muscle cell differentiation are bound predominantly by E2f3b, explaining the unique role of E2f3b in differentiation in this tissue type. Since previous reports of genomic binding patterns for E2Fs, which focused predominantly on E2F1 and E2F4, revealed largely overlapping targets in a number of tissue types (Conboy et al., 2007; Xu et al., 2007; Blahnik et al., 2010; Lee et al., 2011), this finding demonstrated that E2Fs can exhibit selectivity in the target genes that they bind. However, as this was the first study to investigate E2F3

genomic binding patterns on a large scale, additional studies are therefore required to determine if this feature is unique to E2F3 isoforms, and if E2F3a and E2F3b are also able to target distinct genes in different cell types.

1.5 Regulation of Neurogenesis and Neural Stem Cell Fate Decisions

1.5.1 Neurogenesis in the Telencephalon

The mammalian nervous system contains a wide diversity of neuronal and glial cell types, arranged in complex interconnected patterns. Proper brain development is dependent on the coordination of a number of cellular regulatory processes, working together to ensure that appropriate cell fates are achieved at the correct times and locations (Guillemot, 2005). Key to higher order cognitive functioning in mammals is the telencephalon, a forebrain structure that gives rise to the cerebral cortex dorsally (pallium) and the basal ganglia ventrally (sub-pallium) in the post-natal brain (Guillemot, 2005). The cerebral cortex houses the hippocampus, an essential structure in learning and memory formation, and the neocortex, a six layered structure necessary for such higher functions as motor coordination, sensory perception, and processing of thoughts and emotions (Molyneaux et al., 2007; Bonaguidi et al., 2012). Proper development of these cortical structures is essential for brain function, as abnormal development has been linked to a number of neurodevelopmental and psychiatric disorders, including schizophrenia, epilepsy, bipolar disorder, Tourette syndrome, mental retardation, autism and Down syndrome (Keverne, 1999; Benes and Berretta, 2001; Lewis and Levitt, 2002; Sherr, 2003; Polleux and Lauder, 2004; Kalanithi et al., 2005; Kato and Dobyns, 2005; Levitt, 2005; Sanchez et al., 2011; Piontkewitz et al., 2012).

During cortical development, neural precursor cells divide within the germinal zones

that line the lateral ventricles. The specification of distinct neuronal cell fates occurs in a temporally and spatially regulated manner. Dorsally located NPCs give rise to excitatory glutamatergic projection neurons, which migrate radially through the cortex to generate the six cortical layers (Rakic, 1972; Tan et al., 1998; Ware et al., 1999; Anderson et al., 2002; Gorski et al., 2002), while GABAergic (γ-aminobutyric acid) interneurons are generated primarily from progenitor cells located ventrally in an embryonic structure called the ganglionic eminence (GE). Once committed to a neuronal fate, interneurons migrate tangentially to integrate into the cortex (Wonders and Anderson, 2006). The hippocampus is derived from precursor cells in the medial cortical neuroepithelium (Grove and Tole, 1999). Cortical formation and the identity of projection neuron and interneuron sub-types within each cortical layer is highly influenced by temporal regulation of differentiative divisions within the germinal zones, such that earlier born neurons migrate to deeper cortical layers, and later born neurons end up in more superficial layers (Angevine and Sidman, 1961; Rakic, 1974; Guillemot, 2005) (Figure 1.3). This discovery was one of the earliest indications that cell cycle regulatory processes, in this case the timing of cell cycle exit, was closely linked with cell fate decisions in the developing brain. A number of genetic pathways underlie the promotion of neurogenesis and the specification of neuronal sub-types. The best known regulators of these processes include *Foxg1*, basic helix loop helix (bHLH) proteins such as *Neurogenin 1* (*Ngn1*), *Ngn2*, and *Ascl1*, as well as homeodomain proteins, including *Pax6*, *Nkx2.1*, *Gsx2* and the *Dlx* family (Guillemot, 2005).

Neurogenesis is most active during pre-natal development, with the bulk of cortical neurogenesis in the mouse occurring in a highly orchestrated fashion between days E11.5-E16.5 (Caviness et al., 1999; Molyneaux et al., 2007) (Figure 1.3). Neurogenesis persists,

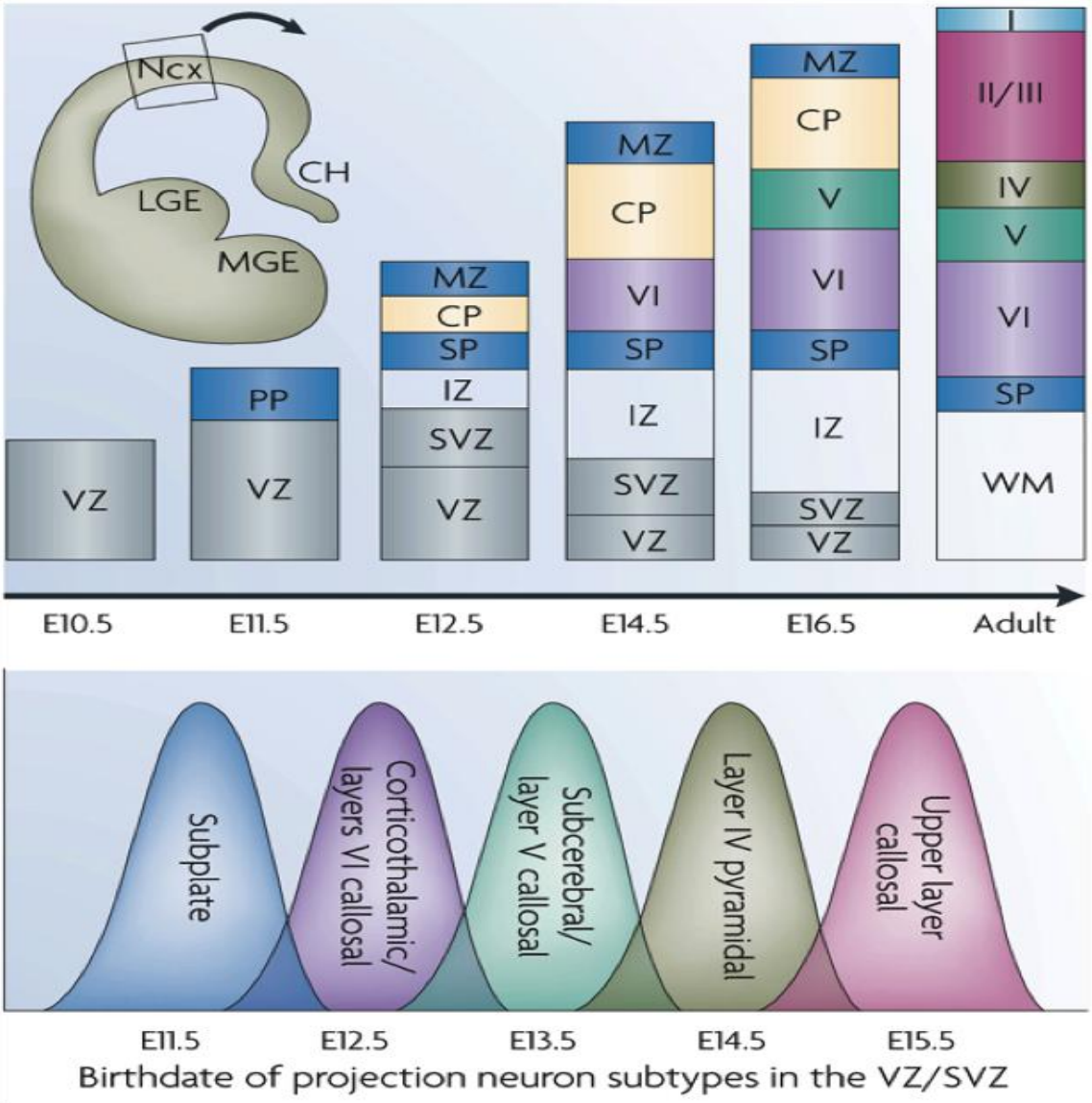


FIGURE 1.3 Neocortex development and birthdates of projection neuron subtypes

At E10.5 the ventricular zone (VZ) is expanding, largely through self-renewing divisions of neural stem cells. Note the appearance of the sub-ventricular zone at E12.5. Neurogenesis and cortical migration takes place predominantly between E11.5-E17.5. The first neurons born form the pre-plate (PP), which becomes separated into the sub-plate (SP) and marginal zone (MZ) by projection neurons born at E12.5, which ultimately form layer VI. Subsequent neuronal cohorts born will sequentially migrate to form the upper cortical layers in an 'inside-out manner', such that deeper cortical layers are born first, and later born neurons migrate past earlier born neurons to form successive cortical layers (layers V, IV, III, and II). One exception is layer I, which derives form the earliest born projection neurons. Ncx = neocortex; CH = cortical hem; LGE = lateral ganglionic eminence; MGE = medial ganglionic eminence; IZ = intermediate zone; CP = cortical plate; MZ = marginal zone; WM = white matter. Adapted from Molyneaux et al., 2007.

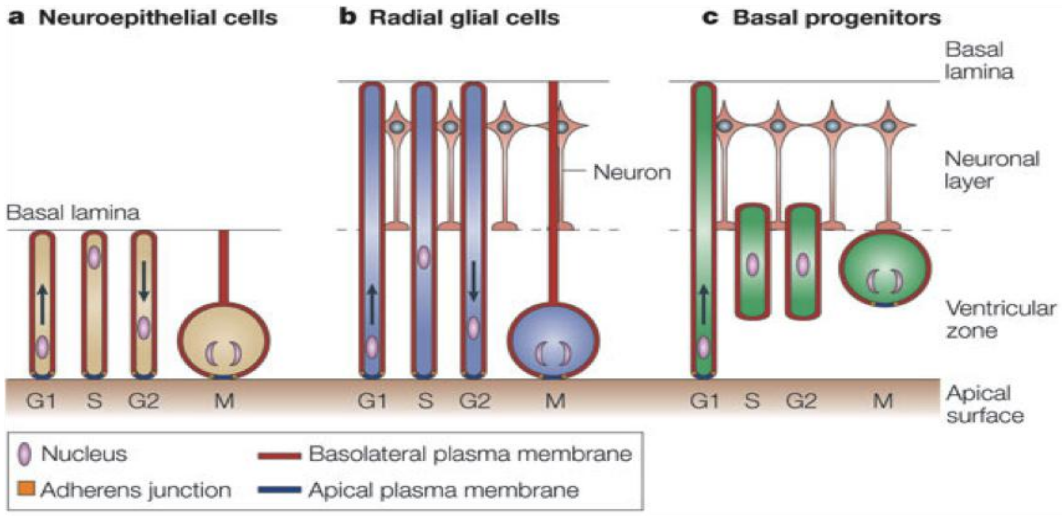
however, in restricted regions in the adult mammalian brain, specifically the sub-granular zone of the dentate gyrus and the olfactory bulb, where newly generated neurons are derived from precursors within the subventricular zone lining the lateral ventricles (Götz and Huttner, 2005; Bonaguidi et al., 2012). The discovery that the adult brain is capable of ongoing neurogensis has indicated an enormous potential in promoting neuronal regeneration after injury by designing novel therapeutics aimed at manipulating neurogenesis-related processes (Reynolds and Weiss, 1992; Lim et al., 2007). Investigating the mechanisms that control the distinct processes underlying neuronal generation and cortical integration, namely stem and progenitor cell divisions, neuronal differentiation and maturation, and cell death, is crucial for understanding the aetiology of brain disorders, the mechanisms underlying brain development, and to facilitate the development of neuronal replacement therapies.

1.5.2 Neural Stem and Progenitor Cells in the Telencephalon

All neurons and glial cells within the mammalian CNS are generated from neural stem cells (NSCs), which derive from a single layer of neuroepithelial cells within the neural tube in early development (Götz and Huttner, 2005). Two defining features of stem cells are the ability to self-renew and multipotency, the ability to differentiate into multiple cell types, in the case of neural stem cells these cell types being neurons, astrocytes and oligodendrocytes. Two types of stem and progenitor divisions are possible: symmetric, in which two cells with identical fates are generated, and asymmetric, resulting in daughter cells with different fates (Molyneaux et al., 2007). Stem cell self-renewal can result from either asymmetric or symmetric divisions, but symmetric divisions clearly lead to a higher rate of self-renewal of the stem cell population.

The process of neurogenesis is highly regulated by a series of symmetric and asymmetric divisions of distinct populations of neural precursor cells, which occur in an ordered fashion to ensure the maintenance of the precursor pool while generating the proper number of cortical neurons (Figure 1.4) (Farkas and Huttner, 2008). During early development neuroepithelial cells, located along the dorsolateral wall of the rostral neural tube where they form the ventricular zone (VZ), undergo extensive symmetric cell divisions to expand their population (Götz and Huttner, 2005; Molyneaux et al., 2007; Farkas and Huttner, 2008). Neurogenesis begins around E11.5 in the mouse, when NPCs in the VZ begin to undergo asymmetric divisions to generate neurons and basal progenitors. At this point, neuroepithelial cells transition to a distinct but highly related cell type, radial glial cells, which express astroglial markers and retain some epithelial characteristics (Huttner and Brand, 1997; Campbell and Götz, 2002; Götz, 2003; Kriegstein and Götz, 2003; Farkas and Huttner, 2008). Radial glial cells eventually replace the neuroepithelial cells, and appear to represent a more fate-restricted type of apical precursor (both neuroepithelial and radial glial cells are classified as 'apical' precursors, residing in the VZ) (Williams et al., 1994; Malatesta et al., 2000; Götz and Huttner, 2005; Molyneaux et al., 2007). As neurogenesis proceeds, a second proliferative layer forms above the VZ, known as the subventricular zone (SVZ) (Bayer et al., 1991). Intermediate basal progenitor cells populate the SVZ, and the majority of their divisions are symmetric in nature, producing two neurons (Farkas and Huttner, 2008). The majority of cortical projection neurons are generated directly from basal progenitor divisions, thus the stem cell-like apical precursors rely heavily on the generation of this intermediate cell type for neurogenesis (Kowalczyk et al., 2009). Neuroepithelial cells are the primary source of neural stem cells (Farkas and Huttner, 2008), however the question

A



B

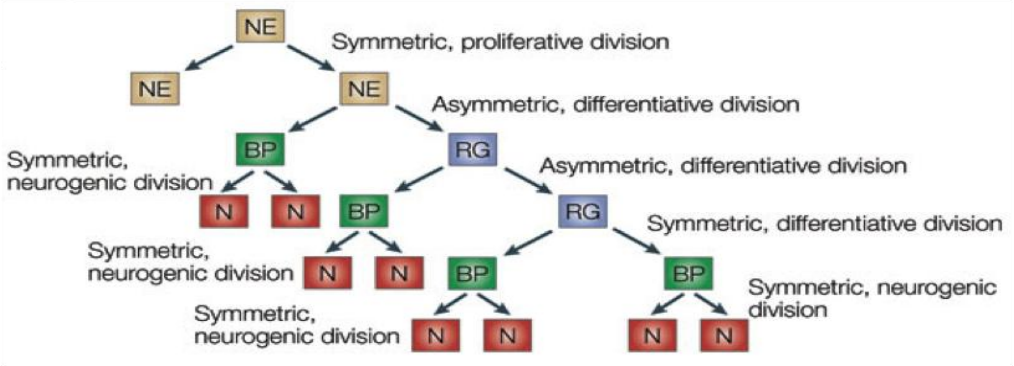


FIGURE 1.4 Polarized features of distinct progenitor cell populations in the ventricular zones and their relative contributions to neurogenesis

(A) Neural stem cells (neuroepithelial and radial glial cells) exhibit interkinetic nuclear migration as they divide in the ventricular zones, whereby the cell nucleus migrates to the basal pole throughout G1, resides basally during S phase, migrates back to the apical end during G2 and divides during mitosis (M) at the apical surface. This process spans the entire apical-basal axis of neuroepithelial cells (a) but ends at the basal boundary of the ventricular zone (VZ/SVZ) in radial glial cells (b). In intermediate basal progenitors (c), nuclear migration is confined to movement to the basal edge of the ventricular zone during G1, and S phase through mitosis occurs basally, concomitant with retraction of the cell from the apical surface and basal lamina, in contrast to neural stem cell populations.

(B) During the process of neurogenesis, neuroepithelial cells (NE) first expand the ventricular zone through symmetric, self-renewing divisions. They then exhibit predominantly asymmetric divisions, at a time when radial glial (RG) characteristics arise, giving rise to a new form of NSC that becomes predominant over NE cells. RG asymmetric divisions give rise to intermediate basal progenitors (BP) and the majority of neurons (N) are produced via symmetric, differentiative BP divisions. Modified from Gotz & Huttner, 2005.

of whether radial glial cells can also be considered neural stem cells is highly debated. The extent of multipotency of radial glial cells is unclear, however imaging studies have shown that they undergo several consecutive self-renewing divisions at the apical ventricular surface (Noctor et al., 2001; 2004; 2008), and they share common structural and niche-dependent features with neuroepithelial cells, as discussed below. Putative NSCs within the SVZ and hippocampal SGZ in the adult brain, cells with the ability to generate neurons and glia post-natally, maintain many features of embryonic radial glial cells. Both express similar markers, such as the intermediate filament protein Nestin and the pluripotency factor Sox2, and NSCs in the adult brain express the glial markers GFAP and GLAST. Radial glial cells will thus be referred to as neural stem, or 'stem cell-like', cells in this thesis, with the understanding that their stem cell potential may be limited compared to other populations. Alternatively, neuroepithelial and radial glial cells will also communally be referred to as apical precursors/ progenitors. Finally, the term 'neural precursor cell' (NPC) will be used to communally refer to both neural stem cells (NSC) and their progenitor derivatives.

1.5.3 Mechanics of Precursor Cell Divisions in the Ventricular Zones

Apical precursors are highly polarized along their apical (proximal to the lateral ventricle) – basal axis (Huttner and Brand, 1997; Wodarz and Huttner, 2003), and are associated with the apical surface of the ventricular zone and with the basal lamina through contacts such as adherens junctions and integrin receptors (Aaku-Saraste et al., 1996; Weigmann et al., 1997; Corbeil et al., 2001; Wodarz and Huttner, 2003). This polarized structure is linked with the property of interkinetic nuclear migration exhibited by neuroepithelial and radial glial cells during cell division (Figure 1.4) (Götz and Huttner, 2005). As they progress through the cell

cycle, their nuclei migrate toward their basal pole during G1, reside at the basal surface of the VZ/SVZ in S phase, migrate back to the apical surface during G2, and finally undergo division (M phase) at the apical surface. Alternatively, the nuclei of basal progenitors are located at the basal surface of the VZ/SVZ during S, G2 and M phases, which is coincident with the retraction of the cell's connections with the apical surface and the basal lamina (Miyata et al., 2004; Götz and Huttner, 2005). Apical progenitors, on the other hand, maintain their apical and basal connections throughout all cell cycle phases (Miyata et al., 2001; Noctor et al., 2001). These features provide a structural distinction between different progenitor populations in the ventricular zones, and the apical-basal polarity of NSC populations is thought to contribute to their mode of cell division and resulting cell fates (described below in section 1.6.2).

1.6 Mechanisms Regulating Neural Precursor Cell Fate Decisions

The mode of neural stem cell divisions and the resulting cell fates of daughter cells *in vivo* are influenced by specific mechanisms, which range from the expression of key pathways and transcriptional regulatory proteins that modify gene expression programs, cellular orientation within the ventricular zone at the point of division, and the length of specific cell cycle phases. Many proteins and pathways have been implicated in NPC fate regulation, and I will discuss in the following sections select examples of the most well known contributors.

1.6.1 Transcriptional Regulation in Neural Precursor Cells

Sequence-specific DNA binding transcription factors, predominantly from the basic helix-loop-helix (bHLH) and homeodomain families, are heavily implicated in the control of cell

fate regulatory processes via the initiation of gene expression programs. These transcription factors include the pro-neural proteins Ascl1, Ngn1, Ngn2, and Math1, which function to promote neuronal commitment, cell cycle exit and differentiation (Guillemot, 2007); the SoxB1 high-mobility-group (HMG) protein family (Sox1, 2 and 3) which promote maintenance of the precursor state, including self-renewal, while inhibiting neurogenesis (Doe, 2008); and patterning proteins that arise early in development to impart distinct identities along the dorsoventral axis of the neural tube and that later affect NPC self-renewal, expansion and/or differentiation (examples include Pax6, Olig2 and Nkx2.2) (Fu et al., 2002; Guillemot, 2007; Sugimori et al., 2007; Sansom et al., 2009; Klempin et al., 2012). Cell-extrinsic signaling pathways, which transmit signals to NPCs from neighbouring cells (such as astrocytes, neuroblasts, and NSCs) also have established roles in regulating NPC fate decisions. Examples include the Wnt/β-Catenin pathway, an important regulator of both NSC maintenance and neurogenesis (Doe, 2008; Shi et al., 2008; Piccin and Morshead, 2011); the Notch signaling pathway, which maintains the self-renewal state of NSCs and inhibits differentiation via transcriptional regulation by the Hes family of transcription factors, which involves direct repression of pro-neural bHLH proteins (Doe, 2008; Shi et al., 2008); and the Sonic Hedgehog (Shh) pathway, which functions as a morphogen during telencephalon development, required for formation of the ventral telencephalon, and promotes NSC self-renewal and proliferation through the Gli family of transcription factors (Chiang et al., 1996; Machold et al., 2003; Palma et al., 2005; Shi et al., 2008). Growth factor pathways that regulate the proliferative expansion of the NPC pool include the EGF, FGF and TGFβ pathways (Shi et al., 2008). Maintaining the correct balance between NPC maintenance, proliferation and neurogenesis appears to rely on the relative levels of pro-

neural and NPC fate promoting genes, as well as transcriptional modification by epigenetic regulators (Doe, 2008; Sansom et al., 2009). Transcriptional repression by Histone deacetylase (HDAC) complexes is, in fact, required for the ongoing self-renewal of NSCs and to inhibit neuronal differentiation, and levels of histone and DNA methylation are also important for the balance between NSC maintenance and differentiation (Martinowich et al., 2003; Biron et al., 2004; Hsieh et al., 2004; Shi et al., 2008). Epigenetic regulators are also important in the maintenance of NSC self-renewal and their differentiation potential in the post-natal brain; key regulators include the Polycomb protein Bmi1, the Methyl-CpG binding proteins Mbd1 and Mecp2, and the Trithorax protein Mll1, (Molofsky et al., 2003; Zhao et al., 2003; Lim et al., 2009; Martín Caballero et al., 2009). Interestingly, Bmi1 has also been implicated in maintaining NSC self-renewal in the embryonic brain by regulating the pRb-E2f pathway via repression of p21 (Fasano et al., 2009). Many of the pathways and transcriptional regulators described here that impact NSC fate decisions are similarly active in both embryonic and adult NPCs, both SVZ and SGZ derived, furthermore, there is a considerable degree of regulatory interactions and feed-back loops formed between the core factors themselves (Doe, 2008; Shi et al., 2008; Ma et al., 2010). Thus, the dynamic modification of gene regulatory pathways within NPCs constitutes an essential cell fate regulatory network during neurogenesis.

1.6.2 Control of Neural Stem Cell Fate by Mitotic Cleavage Plane Orientation

A defining feature of apical precursor cells in the developing brain is their apical-basal polarity, dependent on the presence of adherens junctions, which mediate cell-cell contacts, at the apical end of the lateral plasma membrane (Götz and Huttner, 2005; Farkas and

Huttner, 2008). Extensive evidence suggests that proteins involved in maintaining the integrity of adherens junctions are key regulators of apical precursor cell fate. These include proteins involved in the transport of Cadherins to adherens junction sites, such as Dlg5, N-cadherin itself, and the Notch inhibitor Numb (Kadowaki et al., 2007; Nechiporuk et al., 2007; Rasin et al., 2007), and proteins directly localized to adherens junctions, including Par-3, Par-6, aPKC, the Rho-GTPase Cdc42, β-Catenin, the kinase Akt1 and the phosphatase Pten (Cappello et al., 2006; Imai et al., 2006; Goldstein and Macara, 2007; Narbonne and Roy, 2008). The cell fate of dividing apical precursors is thought to depend on the pattern of distribution of these apical molecular constituents upon cell division (Huttner and Brand, 1997; Wodarz and Huttner, 2003). Thus, a hypothesis has emerged that the mitotic cleavage plane, dictated by the positioning of centrosomes, determines whether an apical precursor cell will undergo an asymmetric or symmetric division (Doe, 2008; Farkas and Huttner, 2008). It has been demonstrated that cleavage planes that bisect the apical plasma membrane (a vertical plane of division relative to the lateral ventricle) are highly likely to result in a symmetric division generating two identical apical precursors, as both daughter cells will inherit apical plasma membrane components. Alternatively, cleavage planes that bypass the apical plasma membrane will result in an asymmetric division, generating daughter cells with different cell fates due to the uneven distribution of cell fate constituents (Huttner and Brand, 1997; Kosodo et al., 2004). Further supporting these observations, altered expression *in vivo* of a number of proteins involved in mitotic spindle assembly has been shown to lead to defects in brain development that suggest disruptions in neural stem cell fate decisions (Doe, 2008; Farkas and Huttner, 2008).

1.6.3 Cell Cycle Regulation Influences Neural Precursor Cell Fate

A number of observations have demonstrated that different aspects of cell cycle regulation are also correlated with NPC fate decisions. In addition to the finding that the timing of cell cycle exit during development predicts the cortical destination of newly born neurons (Angevine and Sidman, 1961; Rakic, 1974; Guillemot, 2005), it was also demonstrated that the decision to adopt a specific neuronal fate is made during the G2 phase of the cell cycle (McConnell and Kaznowski, 1991; Durand and Raff, 2000). Subsequently, it was shown that the fate of neural precursors is influenced by the length of distinct cell cycle phase, with precursors committed to generating fate-restricted basal progenitors or undergoing neuron production exhibiting a substantially longer G1 phase, and apical and expanding basal progenitors having a longer S-phase than those committed to neurogenesis (Takahashi et al., 1995; Calegari et al., 2005; Arai et al., 2011a). Analysis of gene expression programs in expanding and committed neural progenitors demonstrated a differential expression of genes involved in cell cycle regulation, chromatin remodeling, DNA replication and repair, and maintenance of the stem cell state (Arai et al., 2011a). Thus, cell cycle regulation appears to be closely linked with the control of neural precursor cell fate programs.

1.7 The pRB-E2F Pathway is an Important Regulator of Diverse CNS Functions

The phenotypes observed in *Rb1-/-* mouse studies revealed a potentially important role for pRB in the CNS, at the very least in regulation of neuroblast proliferation. Supporting this possibility, examination of pRb expression levels across a panel of mouse tissues demonstrated that, although it is detected in all tissues, its expression was strikingly highest in embryonic liver and brain, with expression in brain remaining high in adult mice

(Bernards et al., 1989). Subsequently, our group observed a classical pattern of pocket protein expression in proliferating and differentiating neural precursor cells (NPCs), where pRB is expressed in both dividing and differentiating NPCs, p107 is high in cycling precursors and rapidly disappears at the onset of differentiation, and p130 is exclusive to non-cycling cells (Callaghan et al., 1999), suggesting that pocket protein family members may regulate unique cell populations in the brain.

1.7.1 *In Vivo* Functions of pRB in the CNS

A pair of studies from our group reported conditional deletion of *Rb1* within the nervous system, where recombination was targeted specifically within the developing telencephalon by intercrossing mice containing an *Rb1* allele in which exon 19 is flanked by loxP sites (Marino et al., 2000) with mice containing a *Cre* recombinase gene knocked into the *Foxg1* locus (Ferguson et al., 2002). This system allows for conditional deletion of *Rb1* within the telencephalon since *Foxg1* expression is restricted to the anterior neural plate and the developing telencephalon (Hébert and McConnell, 2000). As with the *Rb1* knockouts with WT placenta, wide-spread aberrant apoptosis was not observed in conditional knockouts, although survival of Cajal-Retzius neurons was specifically affected (Ferguson et al., 2005). However, these animals exhibited ectopic proliferation of cells specifically outside of the ventricular zones, suggesting a role for pRb in controlling cell cycle exit of differentiating NPCs. Ectopically dividing cells surprisingly expressed early markers of neuronal differentiation, demonstrating a role for pRb in coordinating cell cycle exit and the initiation of differentiation. These phenotypes lead to increased cellularity, enhanced neurogenesis, and a partially penetrant defect in cortical morphology and larger, protruding telencephalic

lobes (Ferguson et al., 2005). Knockout animals die shortly after birth due to respiratory problems, thought to be due to apoptosis within the pons, which also exhibited some recombination (Ferguson et al., 2002). Furthermore, demonstrating a role for pRb in regulation of phenotypes following neuronal commitment, telencephalic specific *Rb1-/-* mice also exhibited defects in cortical laminar patterning and neuronal migration (Ferguson et al., 2005). Specifically, the normal distinction between the intermediate zone and cortical plate based on unique neuronal sub-type markers is absent in the knockouts, thought to be due to defective radial migration of dorsally generated projection neurons through the cortex. Additionally, inhibitory GABAergic interneurons derived from the ventral telencephalon are unable to complete migration to the dorsal cortex, a defect attributed to cell autonomous loss of pRb (Ferguson et al., 2005).

Conditional deletion of of *Rb1* in Nestin expressing cells of the developing CNS, PNS and lens also demonstrated inappropriate cellular proliferation, however extensive apoptosis was observed in the PNS (specifically the dorsal root and trigeminal ganglia) and lens (MacPherson et al., 2003; Chen et al., 2004a). pRb is required for the survival of specific retinal cell types (ganglion, bipolar and rod photoreceptor cells), and its acute loss in terminally differentiated cortical neurons has recently been demonstrated to result in apoptotic death of this cell type (MacPherson et al., 2004; Andrusiak et al., 2012). Furthermore, *Rb1* deletion in precursor cells within the cerebellum caused both increased proliferation and apoptosis specifically of granule cell precursors (Marino, 2003). Thus, the effects of *Rb1* deficiency in the nervous system are context and cell-type specific, particularly with regards to cell survival. Together, these *in vivo* studies provided compelling evidence that pRB plays an important role in multiple processes in the brain that affect

neuron generation and function, ranging from the regulation of cell cycle arrest, differentiation and cell survival, to the migratory potential of committed neurons.

1.7.2 *In Vivo* Functions of p107 and p130 in the CNS

The pocket protein family members p107 and p130 are also implicated in nervous system function. p130 has been linked to the repression of cell cycle entry in retinal interneurons, functioning in a similar manner to pRB, as one copy of either *Rb1* or *Rbl2* is sufficient to prevent ectopic proliferation of this cell type (Ajioka et al., 2007). Additionally, both p107 and p130 are able to functionally compensate for the loss of pRb, as combined deficiency of pRb and either p107 or p130 leads to the development of Retinoblastoma tumours in mice (Robanus-Maandag et al., 1998; MacPherson et al., 2004). As further evidence of pocket protein compensation in the CNS, deficiency of p107 worsens the proliferative and apoptotic defects in cerebellar granule cells caused by pRb deficiency in the cerebellum (Marino, 2003).

Knockout mouse models have also revealed a unique role for p107 in the telencephalon, unrelated to pRb function. p107 deficient mice produce significantly more proliferating progenitors within the VZ/SVZ during development and display an increased capacity for stem cell self-renewal, which results in an expanded precursor population in adulthood (Vanderluit et al., 2004). The increased precursor pool correlates with a defect in the ability of neural precursor cells to commit to a neuronal fate and initiate differentiation, resulting in decreased cortical neurogenesis (Vanderluit et al., 2007a). These findings revealed that p107 functions in a manner quite distinct from pRb by regulating the activity of uncommitted neural precursor cells in the embryonic and adult mouse brain, while pRb

activity is restricted to controlling cell cycle exit in cells that are committed to differentiate (Ferguson et al., 2002). These surprising findings expanded the repertoire of cellular functions known to be regulated by pocket proteins, and revealed distinct requirements for pRB and p107 in the CNS.

1.3 Evidence for E2F Function in the Nervous System

Some of the earliest evidence to suggest that E2Fs may play an important role in nervous system development came from an *in vivo* analysis of the expression patterns of E2f family members during mouse development (Dagnino et al., 1997). E2f1, 2 and 5 were detected in the forebrain starting from E9.5, and became localized to proliferative neuroepithelial ventricular zones throughout the brain and upper spinal cord, with peak expression at E13.5. This expression pattern suggests that increasing neurogenesis and migration out of the ventricular zones is associated with down-regulation of these E2Fs. E2f3 and E2F4 were first detected at E10.5 and became distributed in both proliferative and non-proliferative regions. Specifically, they were detected in the ventricular, intermediate and marginal zones throughout the brain and spinal cord, as well as within the dorsal root ganglia starting around E14.5, when extensive neuron differentiation has occurred in this region. Expression patterns are similar in the developing retina, where E2f1, 2 and 5 are expressed in the retinoblastic layer, which contains undifferentiated cells, throughout retinal development; alternatively, E2f3 and E2f4 are expressed in the ganglion cell layer, where differentiated cells reside, and E2f4 but not E2f3 is present in the retinoblastic layer. These differential expression patterns among E2fs suggest potentially unique functions for different family members in nervous system development.

Studies of pocket protein knockout mice also highlighted an important role for E2fs in controlling diverse NPC and neurogenic cell fates. This is evidenced by findings that loss of either *E2f3* or *E2f1* in *Rb1-/-* telencephalon is able to rescue the cell cycle exit and laminar patterning defects, and can partially rescue the neuronal apoptosis, associated with pRb loss (Mcclellan et al., 2007). Demonstrating a unique role for E2f3, only loss of *E2f3* but not *Ef21* rescues the neuronal migration defects caused by pRb deficiency. In the retina, pRb controls cell division and survival of rod, bipolar and ganglion neurons through E2f1, but E2f2 was surprisingly shown to be required and sufficient for apoptosis and to suppress cell cycle entry in cone photoreceptor cells (Chen et al., 2007; 2013). Additionally, pRb promotes differentiation of a subset of cholinergic interneurons by inhibiting E2f3a activity (Chen et al., 2007). Our group has further demonstrated that both E2f3 and pRb control expression of the *Neogenin* gene in the context of neuronal migration (Andrusiak et al., 2011), and that E2f3 and p107 cooperate to control *Fgf2* expression in proliferating neural precursors (Mcclellan et al., 2009). p107 is not normally associated with E2F1-3, but has been shown to bind these factors in the absence of pRB (Lee et al., 2002); our findings in NPCs have suggested that p107 and E2F3 can actually interact under physiological conditions, perhaps on a target gene specific basis (Mcclellan et al., 2009). Remarkably, the enhanced self-renewal and reduced neuronal commitment in p107 deficient brain can be rescued by inhibition of the Notch1 signaling pathway (Vanderluit et al., 2007a), which is a fundamental pathway in the maintenance of the precursor cell state and promotion of stem cell self-renewal (Hatakeyama et al., 2004; Hitoshi et al., 2002; Ishibashi et al., 1995; Ohtsuka et al., 2001) (Ishibashi et al., 1995; Kageyama and Ohtsuka, 1999; Hitoshi, 2002; Hatakeyama, 2004). These findings provided strong evidence that the pRB pathway may control distinct

neuronal cell fates by regulating expression of cell cycle independent genes through the regulation of E2F transcriptional activity.

Diverse roles have also been reported for E2F family members in the nervous system independently of pocket protein deficiency. E2f1 deficient mice exhibit reduced precursor proliferation in the adult brain, resulting in neurogenic defects in the dentate gyrus and olfactory bulb (Cooperkuhn et al., 2002). Alternatively, E2f1 deficiency leads to cerebellar atrophy and cell cycle re-entry of mature cerebellar and neo-cortical neurons *in vivo* (Cooperkuhn et al., 2002; Wang et al., 2007), demonstrating that E2f1 functions as a cell cycle suppressor in mature neurons. In precursor cells of the developing retina, loss of E2f1 alone, or of all three activator E2Fs (E2f1-3), results in a slight decrease in proliferation (Chen et al., 2009a); however, E2f1-3 are required for the survival of retinal progenitor cells, marked by activation of p53-dependent apoptosis in their absence. Loss of E2f4 leads to striking phenotypes in the developing forebrain. *E2f4* knockouts, which outwardly exhibit severe craniofacial defects, suffer from a loss of ventral forebrain structures concomitant with decreased expression of ventral homeodomain patterning genes, and aberrant expression of dorsal patterning genes (Ruzhynsky et al., 2007). They also have a specific deficiency in the number of self-renewing NPCs, which exhibit decreased self-renewal potential, but their ability to proliferate and differentiate into neuronal or glial lineages was unaffected. Surprisingly, the telencephalic patterning and self-renewal defects were attributed to defective regulation of the Sonic Hedgehog Signaling (Shh) pathway, identifying another novel, cell cycle independent mechanism of E2F function.

1.7.4 An Important Regulatory Role for E2F3 in Telencephalic Neural Precursor Cells

Our laboratory recently discovered an important role for E2f3 in regulating neural stem cell self-renewal and progenitor proliferation, and the phenotypes observed intriguingly suggested opposing functions for E2f3 within the NPC population. More specifically, E2f3 deficient mice have fewer proliferating precursor cells lining the lateral ventricles in embryos and adults (Mcclellan et al., 2007; 2009), and this does not appear to be due to increased cell death. It was also observed that *E2f3-/-* embryos posses more self-renewing precursor cells derived from the ventral telencephalon, and these cells exhibit increased self-renewal potential compared to WT cultures (K.A. McClellan and R.S. Slack, unpublished observations; Appendix A). The increased self-renewal potential, but overall decreased size of the progenitor pool in *E2f3-/-* mice suggested distinct functions for E2f3 in NPCs, and we hypothesized that these may be due to differential functions of E2f3 isoforms. The individual functions of E2f3a and E2f3b in regulating neurogenesis and NPC fate decisions in the developing forebrain have not been directly investigated, and a central theme in this thesis is the determination of the relative roles of E2f3a and E2f3b in these processes. As E2Fs are the ultimate effectors of the core cell cycle regulatory pathway, and E2F3 has been linked to diverse functions in the brain, understanding the regulatory mechanisms by which E2F3 functions in NPCs, as well as the diversity of its target genes, will provide important insight into the mechanisms by which cell cycle control and cell fate decisions are coordinated in the CNS.

1.8 Statement of Objectives

Extensive evidence now exists that the pRB-E2F pathway is involved in controlling diverse cellular functions, both related to and distinct from cell cycle regulation. *In vitro* studies have additionally demonstrated an important role for this pathway in regulating a number of processes in the developing and adult brain, including cell fate decisions within neural precursor cells. Initial studies suggest that E2Fs, predominantly E2F3, can influence these processes by directly regulating novel, cell cycle independent target genes; however, whether this phenomenon is limited to a small number of target genes or represents a wide-spread feature of E2F function is currently unknown. Additionally, E2F3 has been demonstrated to regulate neural stem cell self-renewal and expansion in a differential manner, suggesting unique roles for E2F3 isoforms in stem cell function. The relative contributions of E2F3a and E2F3b to NPC regulation in the brain, at both the phenotypic and gene regulatory level, have not been investigated. As the pRB-E2F pathway has emerged as a clear regulator of NPC fate decisions, and has been implicated as a regulator of other stem cell populations (Wenzel et al., 2007; Sage, 2012), understanding the roles of E2F3 isoforms in NPCs will provide important insight into the mechanisms that both promote and inhibit the precursor cell fate. The research objectives of my PhD studies were as follows:

1. To examine the relative roles of E2f3 isoforms in regulating neural precursor cell fates in the developing brain;
2. To determine the molecular mechanism by which E2f3a and E2f3b differentially regulate neural precursor self-renewal and proliferation versus neurogenesis;
3. To identify the target genes bound by E2f3a, Ef23b and E2f4 in neural precursor cells, in

order to understand the potential extent of E2f regulated functions in NPCs and to determine if E2fs bind unique sets of target genes.

Hypotheses and Summary of Results

In Chapter 2, we tested the hypothesis that ***E2F3 isoforms differentially regulate neural stem cell self-renewal and expansion of the progenitor pool***. As E2F3b, but not E2F3a, has features indicative of a transcriptional repressor, and E2F3a has previously been shown to function as an activator of transcription and cellular proliferation, we expected that E2F3a would promote NPC proliferation, while E2F3b would repress self-renewal. Using mouse models in which either *E2f3a* or *E2f3b* are absent, we uncovered the surprising finding that loss of *Ef23a* results in increased NPC self-renewal while loss of *E2f3b* leads to decreased progenitor proliferation. Additionally, E2F3 isoforms differentially regulated the balance between NPC maintenance and neurogenesis, as loss of *E2f3a* also reduced neurogenesis and loss of *E23fb* increased neuronal generation *in vitro* and *in vivo*. These findings demonstrated that E2F3a inhibits while E2F3b promotes a neural precursor state, contrary to our expectations.

Stemming from these findings and our previous observations that E2Fs can regulate the expression of cell cycle independent genes in the brain, we wondered if E2F3 regulates NPC fate decisions by directly controlling the expression of key cell fate regulatory genes. We observed aberrant expression of the pluripotency factor Sox2 in E2f3 deficient NPCs, which could potentially account for the phenotypes observed by *E2f3a* and *E2f3b* deficiency. Thus, in Chapter 2 we also tested the hypothesis that ***E2f3 isoforms influence the balance between maintenance of the NPC pool and neurogenesis by differentially regulating expression of***

Sox2. By examining Sox2 expression levels and the chromatin environment of the *Sox2* promoter in E2f3 isoform deficient mice, and further employing lenti-viral mediated strategies to knock down Sox2 levels in NPCs, we demonstrated that E2F3a is associated with transcriptional repression of *Sox2* while E2F3b is associated with *Sox2* activation. We also demonstrated the important findings that regulation of *Sox2* by E2F3a accounts for the role of E2F3a in self-renewal, and also influences neurogenesis in embryos and adults, leading to a reduction of neurogenesis and cognitive function in the post-natal brain.

Finally, in Chapter 3 we investigated the wide-spread binding patterns of E2F3a, E2F3b and E2F4 at promoters and surrounding regions of all known mouse genes by performing a series of Chip-on-chip experiments in proliferating neural precursor cells. E2Fs have been implicated in regulating a diversity of cell cycle independent process in neural precursor cells, but it is currently unknown if this is primarily due to transcriptional regulation of cell cycle independent target genes. Thus, we tested the hypothesis that ***E2F proteins bind to the promoters of cell cycle independent genes that play fundamental roles in mediating diverse NPC fate decisions***. Additionally, phenotypes of mice deficient in either E2f3a, E2f3b, or E2f4 demonstrate that all three proteins are required for proper regulation of NPC cell fate decisions, but that they regulate the NPC pool in different manners. One possible mechanism for unique E2F functions is regulation of distinct target genes, thus we also tested the hypothesis that ***distinct E2F family members exhibit specificity in the target genes that they bind***. We found that E2Fs bind to the promoter region of a large number of genes whose protein products are key regulators of neural precursor cell fate decisions, including neurogenesis, self-renewal, growth factor signaling, chromatin remodeling, and of course, cell cycle regulation. Additionally, we found extensive

overlap in the binding patterns of the three E2Fs tested, demonstrating that the majority of E2F targets in NPCs are bound by multiple E2Fs. These studies did however reveal a surprising tissue-specificity of E2F3 isoform binding patterns in neural versus muscle precursors, providing the first evidence of largely distinct E2F-dependent transcriptional programs in different cell types.

CHAPTER 2

Lisa M Julian, Renaud Vandenbosch, Catherine A Pakenham, Matthew G Andrusiak, Angela P Nguyen, Kelly A McClellan, Devon S Svoboda, Diane C Lagace, David S Park, Gustavo Leone, Alexandre Blais[*], and Ruth S Slack[*]. (2013). Opposing Regulation of *Sox2* by Cell-Cycle Effectors E2f3a and E2f3b in Neural Stem Cells. *Cell Stem Cell*. 12, 440-452.

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The experiments were performed by LMJ, except for the reporter assay (Fig. 2.3D) and immunohistochemical staining and quantification of adult brain sections (Fig. 2.6 H&I), which were performed by MGA and APN/ RV, respectively. Mouse behavioural analyses were performed in collaboration with DCL through our behavioural core facility. Experiments were conceptualized by LMJ, with assistance from RSS as well as AB. RV provided technical and conceptual assistance relating to *in vivo* analyses. CAP, KAM, APN and DSS provided technical assistance. RSS and AB provided technical training and conceptual insight. GL generated and provided *E2f3a* and *E2f3b* knockout mouse models. The manuscript was written by LMJ, in collaboration with RSS, and all authors contributed to critical review of the manuscript.

Opposing Regulation of *Sox2* by Cell Cycle Effectors E2f3a and E2f3b in Neural Stem Cells

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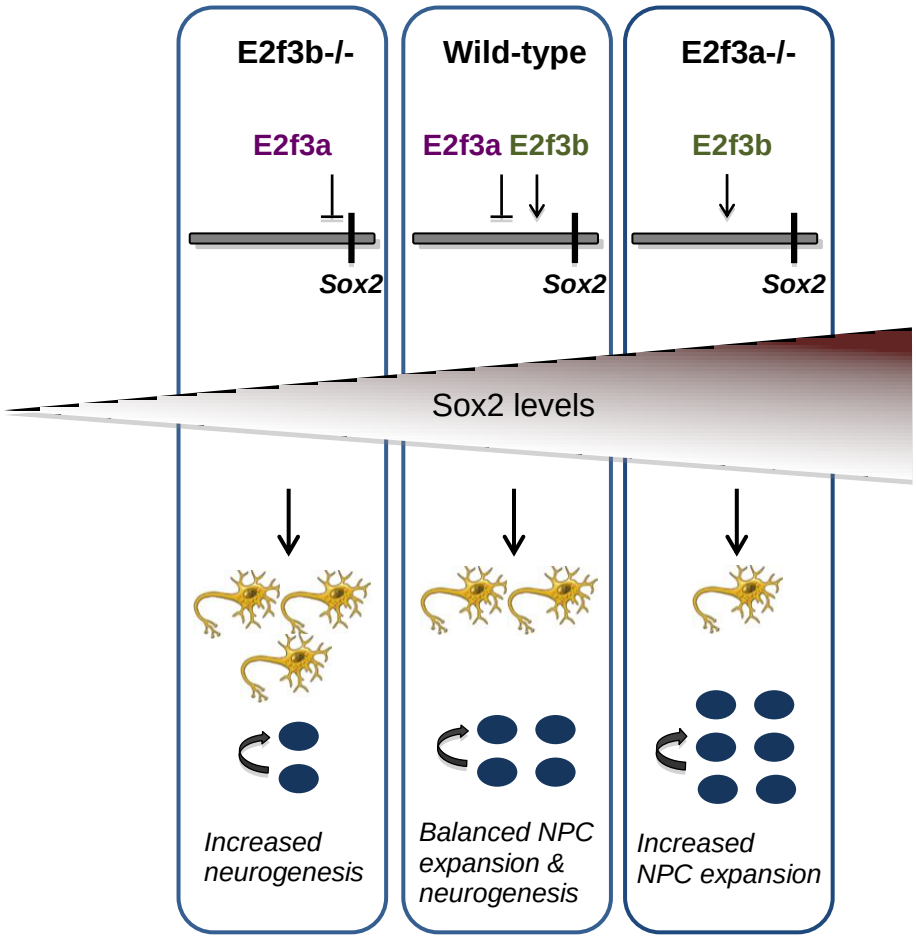
Highlights:

1. The E2f3a&b transcription factors differentially regulate Sox2 levels and cell fate in NPCs
2. Sox2 knock-down restores the elevated self-renewal observed in E2f3a deficient NPCs
3. This novel mechanism of *Sox2* regulation is common in diverse embryonic and adult NPC populations
4. E2f3a deficiency leads to reduced neurogenesis in adulthood and defects in cognitive function

E-TOC: Distinct isoforms of the E2f3 transcription factor differentially regulate *Sox2* expression to modulate precursor cell fate in embryonic and adult neural precursor cells

GRAPHICAL ABSTRACT



SUMMARY

The mechanisms through which cell cycle control and cell fate decisions are coordinated in proliferating stem cell populations are largely unknown. Here, we show that E2f3 isoforms, which control cell cycle progression in cooperation with the retinoblastoma protein (pRb), have critical effects during developmental and adult neurogenesis. Loss of either E2f3 isoform disrupts *Sox2* gene regulation and the balance between precursor maintenance and differentiation in the developing cortex. Both isoforms target the *Sox2* locus to maintain baseline levels of Sox2 expression, but antagonistically regulate Sox2 levels to instruct fate choices. E2f3-mediated regulation of *Sox2* and precursor cell fate extends to the adult brain, where E2f3a loss results in defects in hippocampal neurogenesis and memory formation. Our results demonstrate a novel mechanism by which E2f3a and E2f3b differentially regulate Sox2 dosage in neural precursors, a finding that may have broad implications for the regulation of diverse stem cell populations.

INTRODUCTION

Stem cell fate decisions, such as self-renewal, precursor cell maintenance and commitment to differentiation, have critical outcomes for embryonic development, tissue maintenance, tumour suppression and regeneration. Cortical development depends on a precisely regulated balance of self-renewal within stem cell-like apical precursors (APs), production of rapidly proliferating basal progenitors (BPs) and differentiation of post-mitotic neurons (Englund et al., 2005; Farkas and Huttner, 2008; Hutton and Pevny, 2011) (Fig. 2.1A). Identifying mechanisms that control this balance can inform our understanding of developmental neurogenesis and, more broadly, reveal stem cell biological principles extending to embryonic stem cell (ESC) differentiation, tumour formation, and tissue regeneration.

The pluripotency factor Sox2 is an established regulator of neural precursor proliferation, self-renewal and differentiation during development, and is also required for maintenance of adult stem cell populations in many different tissues (reviewed in (Sarkar and Hochedlinger, 2013)). Over-expression of Sox2 in both mouse and chick embryonic NPCs results in maintenance of the Sox2+ population and defective neurogenesis (Graham et al., 2003; Bani-Yaghoub et al., 2006). Conversely, loss of function of Sox2 in neural precursors leads to precursor loss and reduced or aberrant differentiation, depending on the tissue type and degree of Sox2 loss (Graham et al., 2003; Ferri et al., 2004; Cavallaro et al., 2008; Favaro et al., 2009). Taken together, these studies reveal that the function of Sox2 is strongly influenced by dosage, thus fine tuning of transcription from the *Sox2* locus is crucial for the generation of the correct proportion of precursors versus differentiated cell types. Interestingly, a recent study finds that the Cyclin-dependent kinase inhibitor 1A (p21) binds a *Sox2* enhancer region to regulate Sox2 expression and adult neurogenesis, linking cell

cycle regulation with Sox2-mediated control of neural stem cell (NSC) expansion (Marqués-Torrejón et al., 2013).

Previous evidence suggests that the cell cycle machinery plays a key role in regulating the proliferative expansion and self-renewal capacity of neural precursor cells (NPCs) (Vanderluit et al., 2004; Ruzhynsky et al., 2007; Nishino et al., 2008). However, how specific cell cycle regulatory proteins function in this context remains poorly defined. The Retinoblastoma pocket protein (pRb) family controls cell cycle progression by binding and inhibiting the E2f family of transcription factors. E2fs are classified into the 'activator' subclass, which drive proliferation and transcription, and the 'repressor' subclass, which are thought to repress gene transcription by modifying chromatin structure through association with pocket proteins (Asp et al., 2009). Earlier work has reported that E2f3 is the most highly expressed E2f family member in wild type and pRb deficient neural precursors (Callaghan et al., 1999); suggesting that it may be an important regulator of NPC functions. Understanding how the *E2f3* gene functions to regulate the cell cycle is not entirely straightforward, as the two isoforms (*E2f3a* and *E2f3b*) expressed from its locus have identical domains important for DNA binding, transactivation and pocket protein binding, and only have unique N-termini. Mice lacking both isoforms die perinatally due to cardiac defects (King et al., 2008), while those deficient in either isoform are fully viable (Danielian et al., 2008b; Tsai et al., 2008), suggesting functional overlap. Tissue and cell type specific analysis of pRb and E2f knockout mice suggest that E2f3a is generally a potent activator of transcription and proliferation, while E2f3b weakly induces proliferation and promotes differentiation (Danielian et al., 2008b; Asp et al., 2009; Chong et al., 2009b), but whether

individual E2f3 isoforms make a distinct contribution to developmental and adult neurogenesis is currently unknown.

Here, we use mouse models deficient for either E2f3 isoform to reveal that E2f3a and E2f3b antagonistically regulate Sox2 expression in NSCs. In E2f3b null animals, where E2f3a is the dominant isoform, we find that E2f3a represses *Sox2* in co-operation with the pRb family member p107, reduces precursor self-renewal and promotes differentiation. Conversely, in E2f3a null animals, where E2f3b is the dominant isoform, we find that E2f3b activates *Sox2* expression by recruiting RNA Polymerase II to its promoter, which leads to increased self-renewal and precursor expansion at the expense of differentiation. Knock-down of Sox2 in E2f3a deficient NPCs restored basal levels of self-renewal. Importantly, we find that adult E2f3a null mice have impaired neurogenesis and a reduced capacity for hippocampal-dependent contextual learning, underscoring how the antagonism between E2f3 isoforms is conserved to regulate adult neurogenesis and affect memory formation.

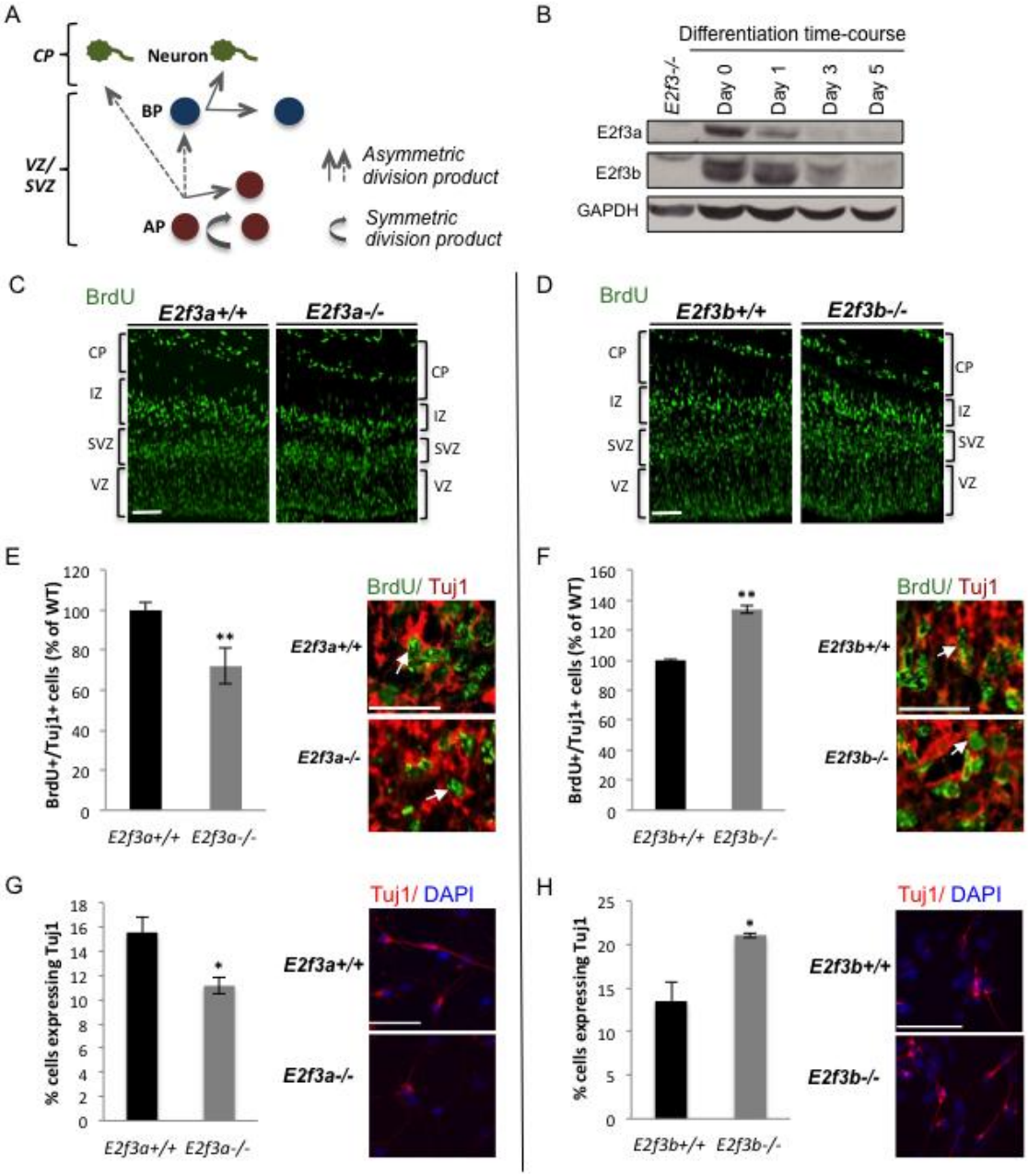
RESULTS

E2f3 Isoforms are Expressed in Neural Precursor Cells

E2f3 is a potent cell cycle regulator and a highly expressed E2f family member in NPCs (Callaghan et al., 1999; Mcclellan et al., 2007), suggesting a potential role for E2f3 in this cell type. Interestingly, we observed that expression of both E2f3 isoforms is enriched in NPCs but reaches negligible levels by day 5 of differentiation *in vitro* (Fig. 2.1B, Supplemental Fig. 2.1A-B), pointing to a regulatory role for both isoforms within the proliferating precursor pool. We asked if E2f3 isoforms play an important role in regulating neural stem and progenitor cell fate decisions by examining mouse lines deficient for *E2f3a* and *E2f3b* (Chen et al., 2007; Tsai et al., 2008).

E2f3a and E2f3b Deficiency Impacts NPC Fate Decisions in an Opposing Manner

We first asked if loss of E2f3 isoforms impacts NPC fate decisions by performing a neuronal commitment assay. Mice were given a single BrdU injection and were sacrificed 24 hours later to visualize BrdU+ cells that had exited the cell cycle and initiated differentiation. There were visibly fewer BrdU+ cells migrating into the subventricular zone (SVZ) and intermediate zone (IZ) of E2f3a-/- mice, but more BrdU+ cells in these regions in E2f3b knock-outs (Fig. 2.1C-D), suggesting a differential commitment to neurogenesis. Newly committed cells that have undergone terminal mitosis can be identified by double labeling with BrdU and differentiation markers, including βIII-tubulin (TuJ1) and Doublecortin (DCX). These BrdU positive cells are also negative for the proliferation marker Ki67. E2f3a-/- mice exhibited a significant reduction in newly committed cells that co-labeled for BrdU/Tuj1 (Fig. 2.1E) or BrdU/DCX (Supplemental Fig. 2.1C), and


A
CP
Neuron
BP
VZ/
SVZ
AP
Asymmetric
division product
Symmetric
division product
B
Differentiation time-course
E2f3-/-
Day 0
Day 1
Day 3
Day 5
E2f3a
E2f3b
GAPDH
C
BrdU
E2f3a+/+
E2f3a-/-
CP
IZ
SVZ
VZ
D
BrdU
E2f3b+/+
E2f3b-/-
CP
IZ
SVZ
VZ
E
BrdU+/Tuj1+ cells (% of WT)
E2f3a+/+
E2f3a-/-
BrdU/ Tuj1
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E2f3a-/-
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Tuj1/ DAPI
E2f3a+/+
E2f3a-/-
H
% cells expressing Tuj1
E2f3b+/+
E2f3b-/-
Tuj1/ DAPI
E2f3b+/+
E2f3b-/-

Figure 2.1. E2f3 Isoforms are Differentially Required for Neuronal Commitment

(A) Cortical development depends on a finely controlled balance of apical precursor (AP) cell proliferation, self-renewal and differentiation. APs can divide symmetrically to expand their population, or asymmetrically to generate one AP and a neuron, glial cell or basal progenitor (BP). BPs generate neurons through asymmetric divisions. VZ = ventricular zone; SVZ = sub-ventricular zone; CP = cortical plate.

(B) Immunoblot for E2f3 in cultured neurospheres induced to differentiate over 5 days. Both E2f3a and E2f3b are expressed in proliferating neurospheres (Day 0), but expression is decreased as differentiation progresses (days 1, 3, and 5). GAPDH was included to ensure equal protein loading.

(C-D) BrdU staining in E14.5 coronal sections following a 24 hour BrdU pulse, to identify cells that have exited the cell cycle. Fewer BrdU+ cells are observed in the E2f3a-/- SVZ/IZ, while more BrdU+ cells are apparent in E2f3b-/-. IZ = intermediate zone; CP = cortical plate.

(E&F) Sections described in panels C-D were immuno-stained for BrdU and Tuj1. The number of BrdU+/Tuj1+ cells was quantified within a defined area through the SVZ/IZ (arrows identify examples of quantified cells). Results are expressed as a percentage of E2f3a+/+ average values +/- SEM (n=4).

(G&H) Neurospheres were expanded *in vitro* and upon first passage were cultured in differentiation media on poly-L-ornithine coated dishes for 3 days, PFA fixed and immuno-stained for Tuj1 and DAPI. E2f3a-/- possesses fewer Tuj1+ cells; E2f3b-/- has more Tuj1+ cells. Results are presented as the percentage of DAPI+ cells expressing Tuj1 +/- SEM (n=4).

For panels E-H, (*$p<0.05$, **$p<0.01$). Scale bars = 50um. See also Supplemental Figure 2.1.

cells that were negative for Ki67 (BrdU+/Ki67-) (Supplemental Fig. 2.1D). In contrast, these same experiments revealed that E2f3b deficient brains contain significantly more committed cells (Fig. 2.1F, Supplemental Fig. 2.1E-F). These results were further supported *in vitro* by quantification of newly committed cells in neurosphere cultures induced to differentiate. Here again, E2f3a deficient NPCs exhibited a reduction in differentiation, while E2f3b deficient precursors had an increase in differentiating cells (Fig. 2.1G-H). Deficiency of either E2f3 isoform does not lead to compensatory expression changes of other pRb/E2f family members (Supplemental Fig. 2.1G-J), demonstrating specificity of E2f3 isoform dependent phenotypes. Thus, deficiency of E2f3 isoforms impacts neural precursor fate decisions in distinct ways, where E2f3a loss reduces, but E2f3b deficiency increases, commitment to a neuronal fate.

To determine if E2f3 isoforms are similarly required to regulate the size of the neural precursor pool in an opposing manner, we quantified the number of proliferating NPCs during forebrain development by performing a 2 hr BrdU incorporation (S phase) and phospho-histone H3 (PH-H3) (M phase) immuno-staining. E2f3a loss resulted in an expanded neural precursor pool (Supplemental Fig. 2.2A&C), specifically affecting the Sox2+ stem-like APs in the VZ (Fig. 2.2A, 2.2C, Supplemental Fig. 2.2E), culminating in a 38% in the size of this population by E17.5 (Fig. 2.2A). Alternatively, loss of E2f3b resulted in an average 25% decrease in precursor numbers throughout development (Supplemental Fig. 2.2B&D), again specifically affecting Sox2 expressing stem-like APs (Fig. 2.2B&D, Supplemental Fig. 2.2F). Concomitant with the expanded precursor population in E2f3a-/- brains, the neuronal output at birth was significantly reduced (e.g., a 24% decrease in later

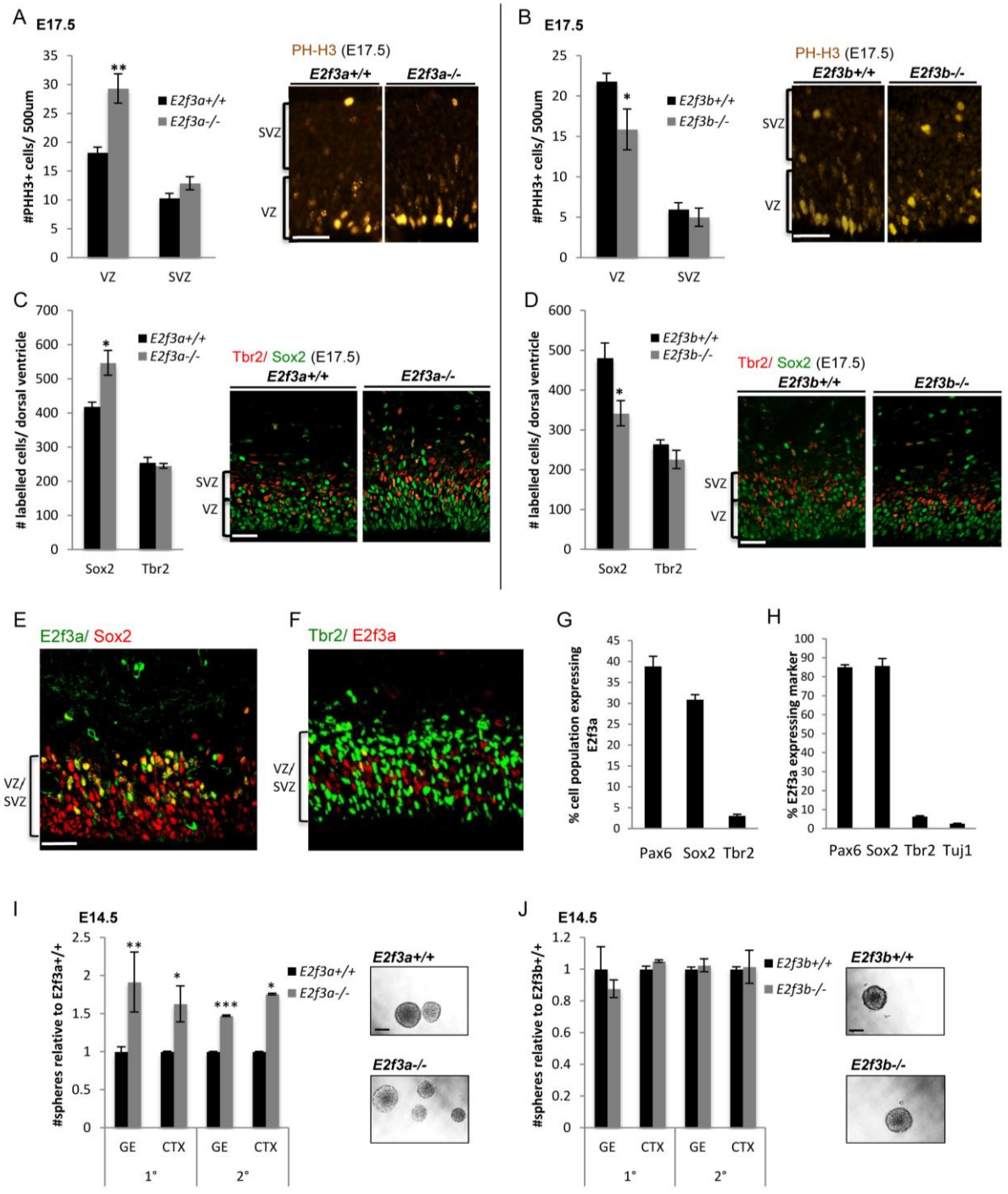
A
E17.5
#PHH3+ cells/ 500um
E2f3a+/+
E2f3a-/-
VZ
SVZ
PH-H3 (E17.5)
E2f3a+/+
E2f3a-/-
B
E17.5
#PHH3+ cells/ 500um
E2f3b+/+
E2f3b-/-
PH-H3 (E17.5)
E2f3b+/+
E2f3b-/-
C
labelled cells/ dorsal ventricle
Sox2
Tbr2
Tbr2/ Sox2 (E17.5)
D
labelled cells/ dorsal ventricle
Tbr2/ Sox2 (E17.5)
E
E2f3a/ Sox2
VZ/ SVZ
F
Tbr2/ E2f3a
G
% cell population expressing E2f3a
Pax6 Sox2 Tbr2
H
% E2f3a expressing marker
Pax6 Sox2 Tbr2 Tuj1
I
E14.5
#spheres relative to E2f3a+/+
GE CTX GE CTX
1° 2°
J
E14.5
#spheres relative to E2f3b+/+

Figure 2.2. E2f3 Isoforms are Differentially Required for Regulation of NPC numbers and Self-renewal

(A&B) PH-H3 staining in E17.5 coronal sections, to label mitotic cells. PH-H3+ cells were quantified along the dorsal surface of the lateral ventricle in either the VZ or SVZ, and numbers were normalized to a defined ventricular length (500um). Quantification demonstrates an expansion in E2f3a-/- and a decrease in E2f3b-/- specifically in the VZ (n=4).

(C&D) Quantification of Sox2+ and Tbr2+ cells within the dorsal cortex at E17.5 demonstrates an increased number of Sox2+ cells in E2f3a-/-, and fewer Sox2+ cells in E2f3b-/- (n=4).

(E) Co-localization of E2f3a (green) with Sox2 (red) in the dorsal cortex (E14.5).

(F) Lack of co-localization between E2f3a (red) and the basal progenitor marker Tbr2 (green) in the dorsal cortex (E14.5).

(G) Quantification of the percentage of all E2f3a+ cells/ section in the dorsal cortex (E14.5) co-expressing Sox2, Pax6, Tbr2 or Tuj1 (n=3).

(H) Quantification of the percentage of Pax6, Sox2, or Tbr2 expressing cells/ section in the dorsal cortex (E14.5) that also express E2f3a (n=3).

(I&J) Increased number of primary and secondary neurospheres in E2f3a-/- precursors derived from both GE and dorsal cortex (CTX) (I); E2f3b knock-outs generate the same number of neurospheres as wild-types (J). Included in the right side of each figure are phase contrast images of neurospheres from the indicated genotypes (n=5-7).

For all panels, results are presented as mean +/- SEM (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Scale bar = 100um. See also Supplemental Figures 2.2-2.4.

born neurons, layers I-III) (Supplemental Fig. 2.3A). In contrast, neuronal output was increased in E2f3b knock-outs (Supplemental Fig. 2.3B). Thus, E2f3a and E2f3b are differentially required to regulate both the expansion of the AP population and their commitment to a neuronal fate.

E2f3 is Expressed in Stem-like Sox2+ Apical Precursors

The impact of E2f3 isoforms on cell fate decisions within the AP population suggests that E2f3 is expressed in Sox2+ precursors. Using an N-terminal E2f3a specific antibody, we detected E2f3a protein within NPCs in the ganglionic eminence (GE), a ventrally located tissue that gives rise to inhibitory interneurons (Wonders and Anderson, 2006), as well as the VZ/SVZ surrounding the lateral ventricle (Supplemental Fig. 2.4A-B). Importantly, E2f3a co-localizes with a subset of Sox2 expressing cells in the GE (Supplemental Fig. 2.4C) and the dorsal cortex (Fig. 2.2E, G-H) (also marked by Pax6 (Supplemental Fig. 2.4D)). Conversely, little E2f3a co-localization was found in committed basal progenitors, which express Tbr2 (Fig. 2.2F-H), or in Tuj1+ neurons (Fig. 2.2H). Quantification of cells co-labeled with E2f3a and cell cycle phase markers revealed that E2f3a is highly enriched in S phase, where 83% of E2f3a+ cells co-expressed BrdU following a 2hr pulse (Supplemental Fig. 2.4E-G). E2f3a expression in S phase precursors supports a role in NPC fate decisions, as a recent study suggests that fate decisions in the developing brain are controlled by gene expression patterns during S phase (Arai et al., 2011b). Thus, E2f3a is expressed predominantly in Sox2+ self-renewing precursors.

E2f3a is Required for Regulation of NSC Self-renewal

We asked next if E2f3 isoforms modulate the self-renewal capacity of the stem cell-like AP population. Loss of E2f3a increased the number of primary and secondary neurospheres generated by both cortical and GE-derived NPC populations by 1.4 to 2 fold at E14.5 (Fig. 2.2I) and E17.5 (Supplemental Fig. 2.4H). Loss of E2f3b, however, showed no effect (Fig. 2.2J, Supplemental Fig. 2.4I). To ask if E2f3a deficiency may affect the mode of AP cell division, we measured the orientation of mitotic spindle poles in control and E2f3a deficient brains. APs undergo mitosis at the apical surface of the lateral ventricle and the orientation of the mitotic spindle pole and cleavage furrow during cytokinesis has been linked with the resulting fate of daughter cells
(Farkas and Huttner, 2008; Godin et al., 2010; Das and Storey, 2012)
(see Supplemental data for detailed methods). In E2f3a knock-outs, we observed 1.5-fold more APs with a cleavage angle within the vertical 75-90° range, associated with symmetric (self-renewing) cell divisions (Supplemental Fig. 2.4J-K). In contrast, there was a corresponding 2.7-fold decrease in the number of divisions within the 0-15° range, suggesting a reduction in asymmetric, differentiative cell divisions. These results suggest that E2f3a deficient brains exhibit an increased proportion of AP cells undergoing symmetric cell divisions, consistent with our *in vitro* studies showing enhanced neural precursor self-renewal.

Opposing Regulation of the *Sox2* Gene by E2f3 Isoforms

To identify target genes through which E2f3 isoforms regulate NPC properties, we performed a genomic ChIP-on-chip screen to identify E2f3 binding sites and associated

target genes in NPCs (L.M.J., Y. Liu, D.S.P., R.S.S., and A.B., unpublished data). From three independent samples of wild-type, E2f3a-/- and E2f3b-/- E14.5 GE neurospheres, we identified the gene encoding the pluripotency factor *Sox2* as a potential target of E2f3 (Fig. 2.3A). Enrichment levels for E2f3 at the *Sox2* promoter were comparable in wild-type, as well as E2f3a and E2f3b deficient cells, indicating that both isoforms bind this locus. Previous studies have shown that changes in Sox2 expression can have dramatic effects on the maintenance and differentiation capacity of neural precursor populations, where elevated Sox2 leads to expansion of the precursor pool and impaired neurogenesis, and decreased Sox2 results in loss of NPCs and dose-dependent defects on neurogenesis (Graham et al., 2003; Bani-Yaghoub et al., 2006; Taranova, 2006; Pevny and Nicolis, 2010). As precursor numbers and neurogenesis are disrupted in E2f3a and E2f3b deficient brains, *Sox2* was a strong candidate to account for these biological effects. We first validated by conventional ChIP that E2f3 binds to the *Sox2* promoter, at an enrichment level comparable to that of previously established E2f3 target genes (Fig. 2.3B). An E2f consensus motif (CTTCCCGC) was identified within the center of the E2f3 binding peak, 371bp upstream of the transcriptional start site (TSS) (Fig. 2.3A), and is conserved in the murine and human genomes. This E2f3 bound region is transcriptionally responsive to E2f3 activity, as indicated by a 2-fold increase in luciferase activity from a *Sox2* promoter fragment (800bp upstream to 285bp downstream of the *Sox2* TSS) following co-transfection of a full length E2f3 construct (Fig. 2.3C-D). Furthermore, point mutations within the E2f consensus motif reduced Luciferase activity by 50% (Fig. 2.3C-D), demonstrating a functional E2f consensus site at 371bp upstream of the *Sox2* TSS.

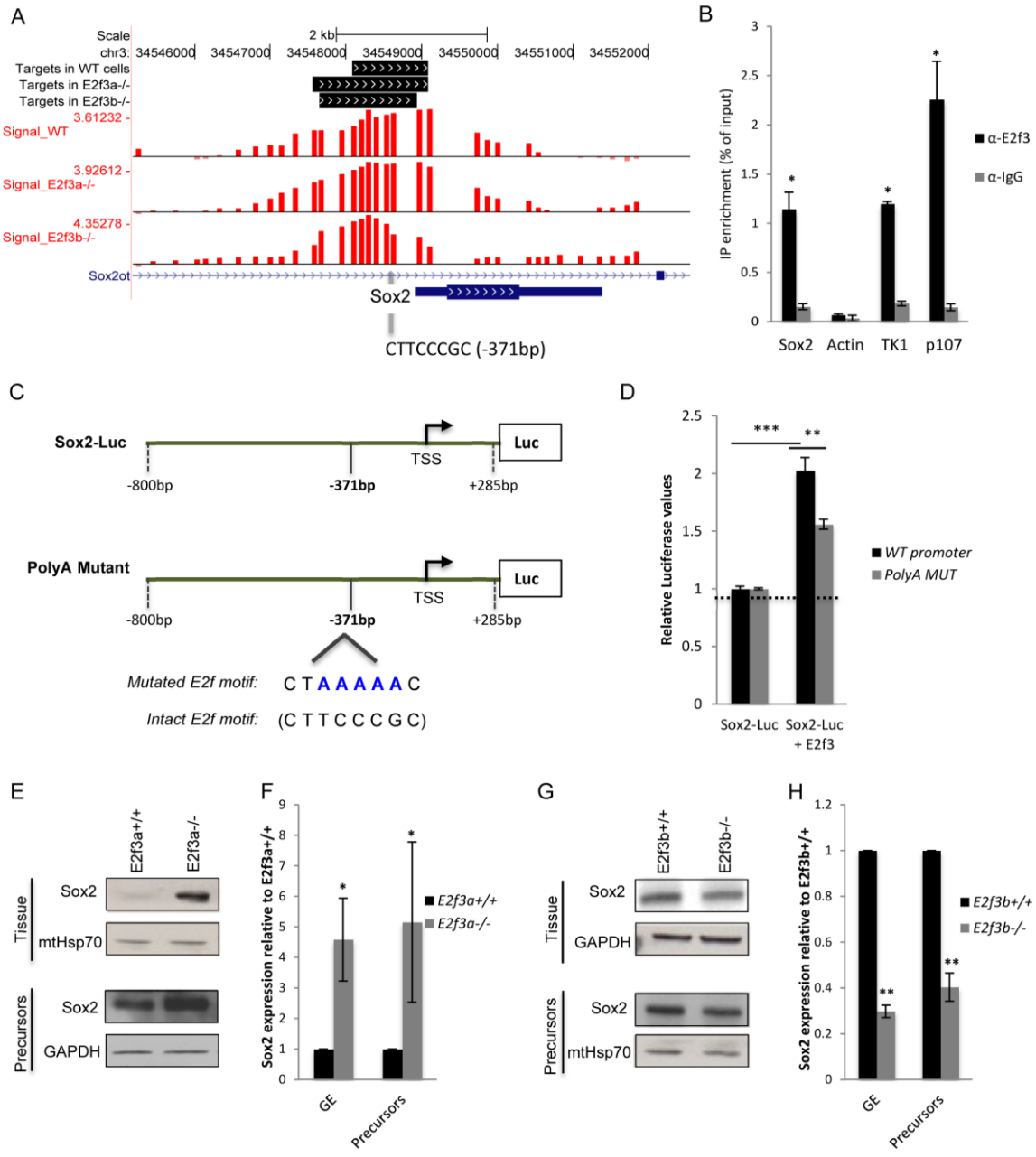
A
Scale
2 kb
chr3: 34546000 34547000 34548000 34549000 34550000 34551000 34552000
Targets in WT cells
Targets in E2f3a-/-
Targets in E2f3b-/-
3.61232 -
Signal_WT
3.92612 -
Signal_E2f3a-/-
4.35278 -
Signal_E2f3b-/-
Sox2ot
Sox2
CTTCCCGC (-371bp)
B
IP enrichment (% of input)
α-E2f3
α-IgG
Sox2
Actin
TK1
p107
C
Sox2-Luc
Luc
TSS
-800bp
-371bp
+285bp
PolyA Mutant
Mutated E2f motif: C T A A A A A C
Intact E2f motif: (C T T C C C G C)
D
Relative Luciferase values
***
**
WT promoter
PolyA MUT
Sox2-Luc
Sox2-Luc + E2f3
E
E2f3a+/+
E2f3a-/-
Tissue
Sox2
mtHsp70
Precursors
Sox2
GAPDH
F
Sox2 expression relative to E2f3a+/+
E2f3a+/+
E2f3a-/-
GE
Precursors
G
E2f3b+/+
E2f3b-/-
Tissue
Sox2
GAPDH
Precursors
Sox2
mtHsp70
H
Sox2 expression relative to E2f3b+/+
E2f3b+/+
E2f3b-/-
GE
Precursors

Figure 2.3. E2f3 Isoforms Regulate *Sox2* Expression in an Opposing Manner

(A) Binding peak profiles for E2f3 from E2f3a+/+, E2f3a-/- and E2f3b-/- ChIP-on-chip experiments, generated with UCSC Genome Browser (http://genome.ucsc.edu/). E2f3 binding peaks extend throughout the proximal promoter region and the TSS. An E2f consensus motif was identified at 371bp upstream of the TSS.

(B) RT-PCR analysis of E2f3 ChIP experiments shows E2f3 binding at the *Sox2* promoter with a similar enrichment value as for other known E2f targets. Plotted is the mean from at least three independent experiments +/- SEM (n=4).

(C) Model for Luciferase experiments. E2f3 dependent activity was tested from a Sox2 promoter fragment covering -800bp to +285bp relative to the TSS. For E2f consensus site mutation, 5 core nucleotides were replaced with Adenine.

(D) E2f3a drives Sox2-Luciferase activity. Mutation of the E2f consensus motif reduced E2f3 mediated transcription by 50% (n=4-6).

(E&G) Immunoblot for Sox2 from cultured E2f3a (E) or E2f3b (G) neural precursors or GE tissue. GAPDH and mtHsp70 were included as protein loading controls.

(F&H) Quantification by densitometry of immunoblots shows that E2f3a knock-outs express significantly more Sox2 (F), while E2f3b knockouts have lower Sox2 levels (H).

For all quantifications, data are plotted as mean +/- SEM (*$p<0.05$, **$p<0.01$, ***$p<0.001$). See also Supplemental Figure 2.5.

To determine if E2f3 isoforms regulate Sox2 levels *in vivo* and *in vitro*, we measured Sox2 protein levels in GE-derived tissue and cultured neurospheres, as NPCs from this region are predominantly Sox2+ (Supplemental Fig. 2.5A-B). We show that E2f3a and E2f3b regulate Sox2 expression in a reciprocal manner. Specifically, E2f3a-/- neurospheres and GE tissue exhibited a 2.3 and 6-fold increase, respectively, in Sox2 levels (Fig. 2.3E-F). In contrast, E2f3b-/- neurospheres and GE tissue express Sox2 at 40% and 30% of wild-type levels (Fig. 2.3G-H). These results suggest an opposing role for E2f3 isoforms in the regulation of the *Sox2* gene.

E2f3a Represses NSC Self-renewal through *Sox2* Regulation

To directly determine if E2f3a represses self-renewal by regulating *Sox2*, we asked if Sox2 knockdown could rescue the enhanced self-renewal phenotype observed in E2f3a deficient cultures. E2f3a+/+ or E2f3a-/- neurospheres were infected with a bicistronic lentivirus expressing GFP and one of two shSox2 or scrambled control sequences. Importantly, each shSox2 construct reduced Sox2 expression in E2f3a-/- cells to a level comparable to GFP infected wild-type cells (Fig. 2.4A-B). shSox2 mediated knockdown of Sox2 in E2f3a-/- cultures restored neurosphere numbers (Fig. 2.4C-D) and self-renewal capacity (Fig. 2.4E) back to basal levels. To ask whether elevated Sox2 can account for the increased self-renewal in E2f3a-/- precursors, we over-expressed Sox2 in wild-type cultures (Fig. 2.4F). Sox2 over-expression in E2f3a+/+ precursors increased self-renewal (Fig. 2.4G) and correspondingly decreased neurogenesis (Fig. 2.4H) to levels observed in E2f3a deficient cells. Furthermore, over-expression of Sox2 in E2f3a-/- precursors, which already express elevated Sox2, did not further increase neurosphere numbers. Thus, E2f3a functions to

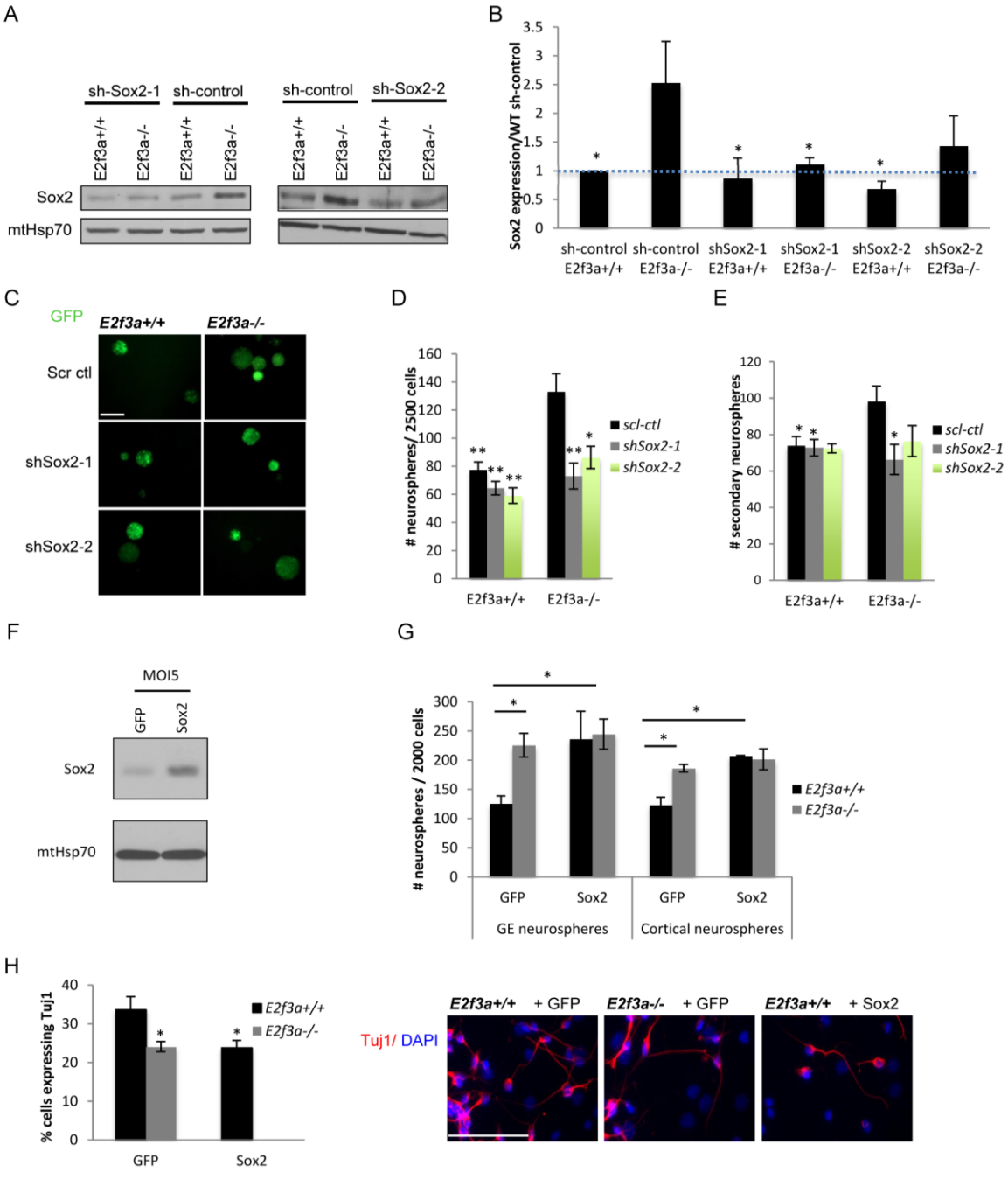
A
sh-Sox2-1
sh-control
sh-control
sh-Sox2-2
E2f3a+/+
E2f3a-/-
E2f3a+/+
E2f3a-/-
E2f3a+/+
E2f3a-/-
E2f3a+/+
E2f3a-/-
Sox2
mtHsp70
B
Sox2 expression/WT sh-control
3.5
3
2.5
2
1.5
1
0.5
0
sh-control E2f3a+/+
sh-control E2f3a-/-
shSox2-1 E2f3a+/+
shSox2-1 E2f3a-/-
shSox2-2 E2f3a+/+
shSox2-2 E2f3a-/-
C
GFP
E2f3a+/+
E2f3a-/-
Scr ctl
shSox2-1
shSox2-2
D
neurospheres/ 2500 cells
160
140
120
100
80
60
40
20
0
scl-ctl
shSox2-1
shSox2-2
E2f3a+/+
E2f3a-/-
E
secondary neurospheres
120
100
80
60
40
20
0
scl-ctl
shSox2-1
shSox2-2
E2f3a+/+
E2f3a-/-
F
MOI5
GFP
Sox2
Sox2
mtHsp70
G
neurospheres / 2000 cells
300
250
200
150
100
50
0
E2f3a+/+
E2f3a-/-
GFP
Sox2
GFP
Sox2
GE neurospheres
Cortical neurospheres
H
% cells expressing Tuj1
40
30
20
10
0
E2f3a+/+
E2f3a-/-
GFP
Sox2
E2f3a+/+ + GFP
E2f3a-/- + GFP
E2f3a+/+ + Sox2
Tuj1/ DAPI

Figure 2.4. Regulation of NSC Self-renewal by E2f3a is Sox2-dependent

(A-B) Immunoblot analysis (A) and densitometry quantification (B) of Sox2 expression in cultured GE neurospheres (E14.5) 5 days post infection (p.i.) with scrambled (Scr) control or sh-Sox2 lentiviruses (n=3). mtHsp70 was included as a measure of protein loading.

(C) Representative images of GFP positive neurospheres 7 days p.i. Scale bar = 200um.

(D&E) Infected cultures were plated immediately for primary neurosphere assays (D) and one week later were used in secondary neurosphere assays (E). Neurosphere numbers are restored in E2f3a knock-outs following Sox2 knock-down (n=4).

(F) Immunoblot demonstrating increased Sox2 expression in neurospheres infected with a Sox2 expressing lentivirus compared to GFP infected cells, 4 days p.i.

(G) Cells were plated 7 days p.i. for secondary neurosphere assays. Sox2 over-expression in wild-type cells increases self-renewal (n=3).

(H) E2f3a+/+ and E2f3a-/- neurospheres were cultured in differentiation media on poly-L-ornithine plates 7 days p.i. and were fixed and stained for Tuj1/ DAPI after 6 days. The percentage of DAPI+ cells that express Tuj1 were quantified. Sox2 over-expression inhibits neuronal differentiation *in vitro*. Scale bar = 50um (n=3).

Significance was determined for all samples compared to E2f3a-/- (panels B, D&E) or E2f3a+/+ (panels G&H) cells infected with control virus. All data are presented as the mean +/- SEM (*$p<0.05$, **$p<0.01$).

maintain Sox2 levels below a specific threshold, beyond which precursor self-renewal and cell fate decisions are markedly disrupted.

E2f3 Isoforms Recruit Distinct Transcriptional Co-factors to the *Sox2* Promoter

To determine the mechanism by which E2f3a and E2f3b antagonistically regulate Sox2 expression we identified the regulatory factors recruited to the *Sox2* locus by each isoform. First, we confirmed that both isoforms bind the *Sox2* promoter, within a 200bp region surrounding the conserved E2f motif ('US', upstream binding site) and at the transcriptional start site (TSS), as E2f3 enrichment is similar in wild-type, E2f3a-/- and E2f3b-/- neural precursors (Fig. 2.5A). Consistent with E2f3b as an activator of *Sox2* expression, in E2f3a-/- cells, where only the E2f3b isoform is present, we observed enrichment of RNA Polymerase II (Pol. II) at and beyond the TSS (Fig. 2.5B) and the trimethyl-H3K4 (H3K4Me3) chromatin modification (Fig. 2.5C), as well as a decrease in trimethyl-H3K27Me3 (Fig. 2.5C). Each of these changes are associated with transcriptional activation, demonstrating that in the absence of E2f3a, Sox2 expression is elevated due to an increased ratio of bound E2f3b/Pol. II complexes. Conversely, binding of the repressive pocket protein p107 was significantly enriched in the absence of E2f3b, where only E2f3a is present, and was decreased in E2f3a-/- cells (Fig. 2.5D). These findings show that E2f3a functions as a repressor at the *Sox2* promoter by recruiting p107. Supporting this conclusion, GE tissue from p107 deficient animals exhibited a 2.2-fold increase in Sox2 levels compared to wild-type controls (Fig. 2.5E).

The percentage of precursor cells in each cell cycle phase was not altered by E2f3a or E2f3b deficiency (Supplemental Fig. 2.6A&B), thus the changes in binding enrichments we

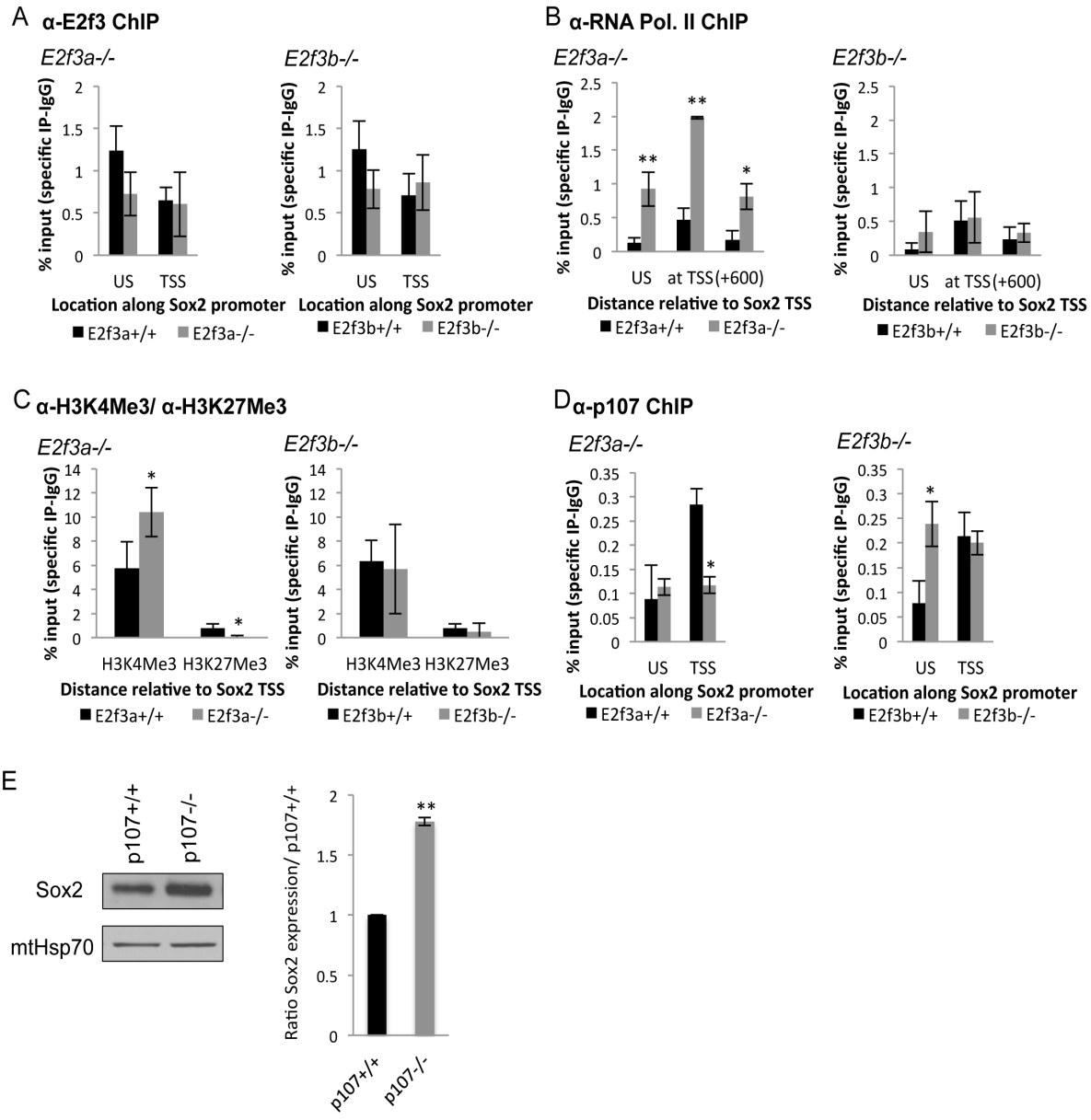
A α-E2f3 ChIP
E2f3a-/-
E2f3b-/-
% input (specific IP-IgG)
US TSS
Location along Sox2 promoter
E2f3a+/+ E2f3a-/-
E2f3b+/+ E2f3b-/-
B α-RNA Pol. II ChIP
US at TSS(+600)
Distance relative to Sox2 TSS
C α-H3K4Me3/ α-H3K27Me3
H3K4Me3 H3K27Me3
D α-p107 ChIP
E
p107+/+
p107-/-
Sox2
mtHsp70
Ratio Sox2 expression/ p107+/+

Figure 2.5. E2f3 isoforms recruit distinct transcriptional co-factors to *Sox2* promoter

(A-D) ChIP was performed in E2f3a-/-, E2f3b-/- and wild-type (both E2f3a+/+ and E2f3b+/+) GE derived neurospheres using antibodies against E2f3 (A), RNA Polymerase II (B), H3K4Me3 and H3K27Me3 (C), and p107 (D). Chromatin enrichment was quantified by RT-PCR using primers designed to amplify 200bp regions centered on either the upstream conserved E2f motif or the TSS of the *Sox2* promoter. For all panels we have plotted values for the specific antibody IP with IgG values subtracted (n=3-5).

(E) Immunoblot analysis and densitometry quantification of p107+/+ and p107-/- GE tissue shows a significant increase in Sox2 levels in the absence of p107 (n=3). See also Supplemental Figure 2.6.

Data for all panels are plotted as the mean +/- SEM (*$p<0.05$, **$p<0.01$).

observed in E2f3 isoform deficient cells could not be explained by disrupted cell cycle dynamics, but truly to altered enrichment of these factors. We show that *Sox2* expression is regulated by E2f3 isoforms in an opposing manner, whereby E2f3a recruits thetranscriptional repressor p107, and E2f3b recruits activator complexes to the promoter. This reveals a novel mechanism for regulation of the pluripotency factor *Sox2*, through the cell cycle effectors E2f3a and E2F3b.

A Common Mechanism of E2f3a-mediated *Sox2* Regulation in Embryonic and Adult NSCs

The requirement for controlled Sox2 expression in NSCs extends from development to adulthood (Ferri et al., 2004; Cavallaro et al., 2008; Favaro et al., 2009; Pevny and Nicolis, 2010), thus we hypothesized that E2f3-dependent *Sox2* regulation is also important in adult NSCs. To evaluate the role of E2f3a in the adult we generated animals containing two modified E2f3 alleles: one *floxed* allele and a second E2f3a deficient allele (E2f3-*flox*/3a-). To induce acute removal of E2f3a, cultured SVZ precursors from adult E2f3-*flox*/3a- animals were infected with a Cre expressing lentivirus, which removes *E2f3a* but leaves *E2f3b* intact (Fig. 2.6A). As with embryonic E2f3a-/- precursors, Cre infected cells exhibited increased neurosphere self-renewal (Fig. 2.6B), and were impaired in their ability to generate neurons (Fig. 2.6C). Importantly, these self-renewal and neurogenic changes were accompanied by increased Sox2 expression (Fig. 2.6D). Furthermore, absence of E2f3a reduced enrichment of E2f3 (Fig. 2.6E) and p107 (Fig. 2.6F), and significantly increased recruitment of RNA Polymerase II (Fig. 2.6G), at the *Sox2* promoter. Thus, NSCs maintain a common mechanism of E2f3a dependent *Sox2* regulation from development to adulthood.

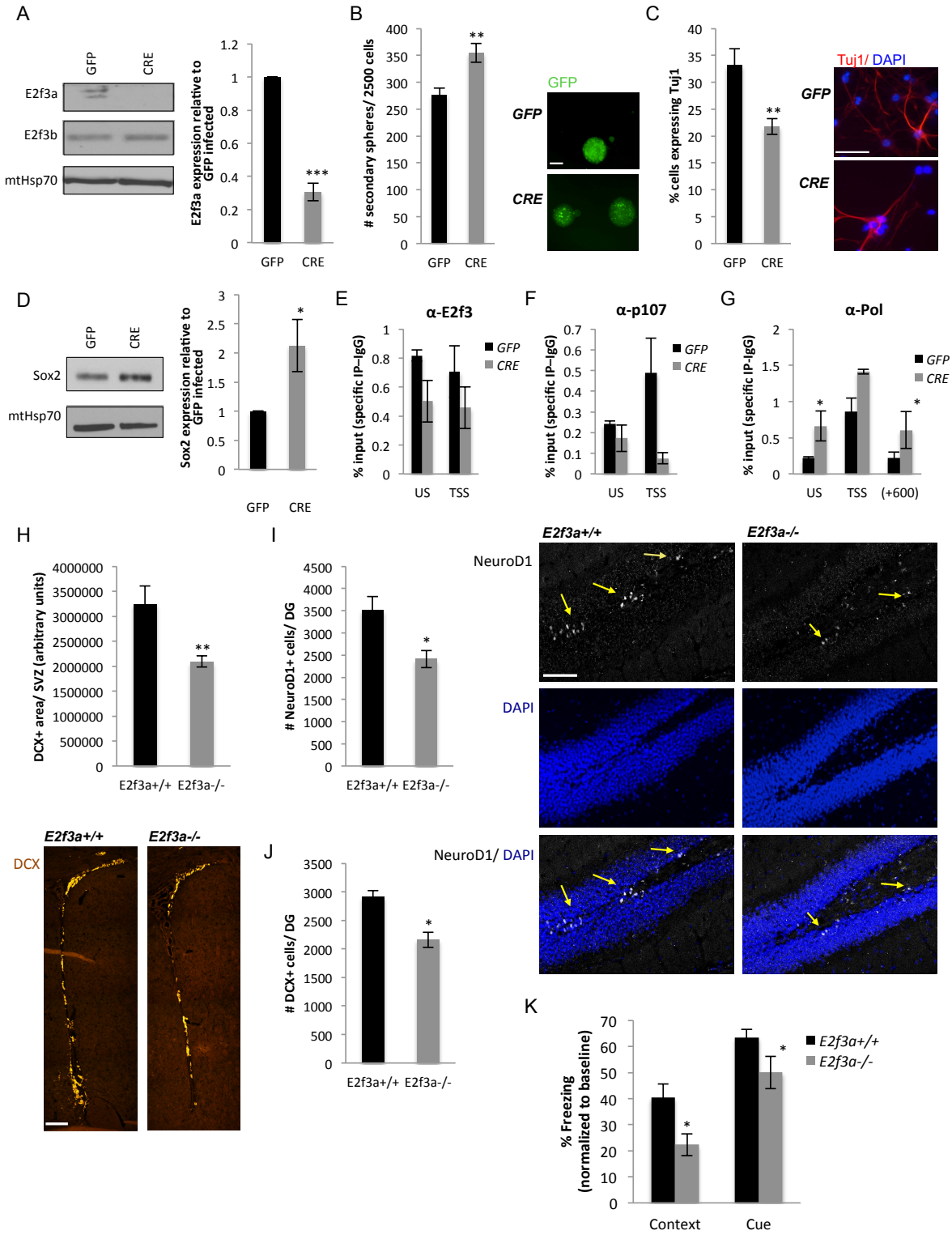
A
GFP
CRE
E2f3a
E2f3b
mtHsp70
E2f3a expression relative to GFP infected
GFP
CRE
B
secondary spheres/ 2500 cells
GFP
CRE
C
% cells expressing Tuj1
Tuj1/ DAPI
GFP
CRE
D
Sox2
mtHsp70
Sox2 expression relative to GFP infected
E
α-E2f3
% input (specific IP–IgG)
US
TSS
F
α-p107
G
α-Pol
(+600)
H
DCX+ area/ SVZ (arbitrary units)
E2f3a+/+
E2f3a-/-
I
NeuroD1+ cells/ DG
NeuroD1
DAPI
NeuroD1/ DAPI
DCX
J
DCX+ cells/ DG
K
% Freezing (normalized to baseline)
Context
Cue

Figure 2.6. E2f3a Regulates *Sox2*, Neurogenesis and Cognitive Function in the Adult Brain

(A) Immunoblot showing loss of E2f3a, but not E2f3b, in E2f3-*flox*/E2f3a- adult SVZ precursors 4 days after Cre infection. Results were quantified by densitometry, using mtHsp70 as a loading control (n=4).
(B) Infected cells were plated for neurosphere assays 7 days p.i. and regenerated neurospheres were counted 6 days later. Cre infected cells generate more neurospheres than controls. Image to the right shows GFP expressing neurospheres infected with either control (GFP) or GFP-CRE virus 7 days p.i. (n=7).
(C) Neurospheres were plated in differentiation media on poly-L-ornithine coated dishes 7 days p.i. and were fixed and stained for Tuj1/DAPI after 6 days. The percentage of DAPI+ cells expressing Tuj1 was quantified. Cre infected cells have a reduced capacity to generate neurons (n=5).
(D) Elevated Sox2 in Cre versus GFP infected E2f3-*flox*/E2f3a- adult precursors (5 days p.i.), quantified by densitometry, using mtHsp70 as a loading control (n=3).
(E-G) RT-PCR analysis of ChIP assays for E2f3 (E), p107 (F) and RNA Polymerase II (G) in GFP or Cre infected E2f3-*flox*/E2f3a-/- precursors (n=4).
(H-L) Neurogenesis was measured in the adult brain by quantifying the total area of DCX staining along the SVZ (n=4) (H), and the number of NeuroD1+ or DCX+ cells in the DG of the hippocampus (n=3) (I-J).
(K) E2f3a-/- mice spent 45% and 21% less time freezing in the context (p=0.015) and after the auditory cue (p=0.047), respectively, following fear conditioning training (n=18 for E2f3a+/+, n=13 for E2f3a-/-).
For all panels, data are presented as mean +/- SEM. Scale bar = 100um for panels B, H & J and 25um for panel C (*p<0.05, **p<0.01, ***p<0.001). See also Supplemental Figure 2.7.

Loss of E2f3a Disrupts Neurogenesis and Cognitive Function in the Adult Brain

Given that E2f3a regulates *Sox2* in both embryonic and adult NSCs, we asked if absence of E2F3a had a functional consequence in the adult brain. We first evaluated the levels of neurogenesis in E2f3a+/+ and E2f3a-/- adult brains in two distinct neurogenic regions, the SVZ and the dentate gyrus (DG) of the hippocampus, where neurogenesis is required, respectively, for olfactory function and hippocampal memory formation. As determined by NeuroD1 and DCX staining, the number of committed neurons was significantly decreased by 35% in the SVZ (Fig. 2.6H) and 31% in the DG (Fig. 2.6I-J), revealing an impairment in adult neurogenesis that has further progressed since late stages of development. We also found that E2f3a-/- mice are significantly impaired in their ability to learn and remember the association between an aversive experience and environment in the classical fear conditioning paradigm (Wehner & Radcliffe, 2004). In this test, animals are trained to acquire a learned response to an aversive stimulus (foot shock) that is associated with a specific environment (context) and a tone (cue). Following training, animals are tested for their ability to have learned that the context or cue is associated with the aversive stimulus by measuring their freezing behaviour during exposure to each condition. E2f3a-/- mice exhibited a significant 45% decrease in freezing behavior associated with amygdala and hippocampal-dependent contextual learning (n-Burgin and Schinder, 2012), while freezing associated with amygdala-dependent cue learning (Wehner & Radcliffe, 2004) was decreased more subtly by 21% (Fig. 2.6K). The reduced freezing in E2f3a-/- versus control mice was not due to differences in the unconditioned freezing behavior, as assessed during training and before tone presentation in the cue trial (Supplemental Fig. 2.7A&B), nor to differences in foot shock threshold (Supplemental Fig. 2.7C). These results suggest that

E2f3a influences the formation of associative memories, and its loss reveals defects in at least two telencephalic structures, with the most severely affected function (contextual learning) being that which is influenced by adult neurogenesis(n-Burgin and Schinder, 2012). Thus, E2f3a is required to regulate neural precursor maintenance and neurogenesis in both the embryonic and adult brain, and this role significantly impacts cognitive function.

DISCUSSION

This study presents two key discoveries. First, we show that E2f3 isoforms play opposing roles in regulating the balance between neural precursor self-renewal and differentiation during developmental neurogenesis. Loss of E2f3a leads to neurogenic defects in adulthood, underscoring the importance of E2f3 in mediating fate choices in both embryonic and adult NSCs. Second, we report a transcriptional mechanism by which E2f3 isoforms antagonistically regulate levels of *Sox2* expression. Alteration of *Sox2* expression by loss of either E2f3 isoform shifts the equilibrium between precursor expansion and differentiation, thereby affecting downstream generation of cortical neurons and ultimately cognitive function.

Based on our findings and previous reports of E2f3 isoform expression patterns (Adams et al., 2000), we predict that E2f3a is predominant during S phase, while E2f3b is expressed throughout the cell cycle. Thus, at different phases of the cell cycle, E2f3a and b isoforms dynamically fine-tune Sox2 expression levels. We present a model of E2f3-dependent *Sox2* regulation in which both E2f3 isoforms, in a see-saw like fashion, regulate levels of Sox2 in proliferating NSCs to ensure the proper balance of precursor expansion and differentiation (Fig. 2.7A). When E2f3b is lost, E2f3a/p107 mediated repression is not balanced by E2f3b mediated activation, leading to lower Sox2 levels and increased neurogenesis at the expense of precursor expansion (Fig. 2.7B). Conversely, E2f3a deficiency leads to activation by E2f3b that is not balanced by E2f3a mediated repression, resulting in elevated Sox2 levels and, consequently, increased precursor self-renewal at

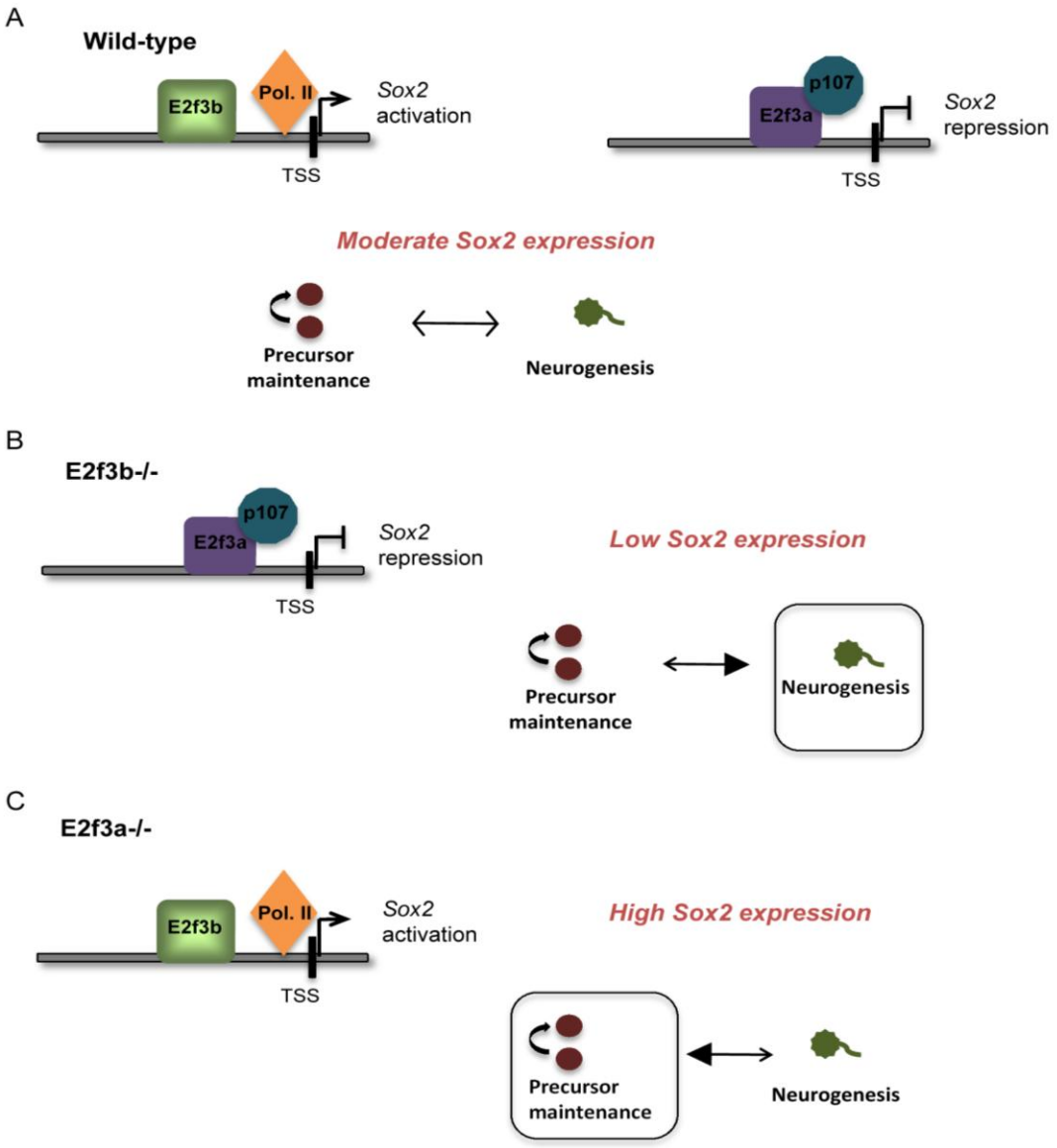

Figure 2.7. Model of E2f3 Function: E2f3 Isoforms Regulate *Sox2* Transcription in an Opposing manner to Direct NSC Fate Choice

(A) In wild-type conditions, E2f3b activates and E2f3a represses *sox2* transcription, allowing for dynamic fine-tuning of Sox2 levels. This fine-tuning maintains the proper balance between neurogenesis and expansion of the NPC pool.

(B) In the absence of E2f3b, E2f3a/p107 mediated repression dominates and reduces Sox2 levels, thereby increasing neurogenesis at the expense of precursor expansion.

(C) In the absence of E2f3a, E2f3b mediated activation is unbalanced by E2f3a repression, thus increasing Sox2 levels and promoting NPC expansion at the expense of neurogenesis.

the expense of neurogenesis (Fig. 2.7C). This functional model illustrates the requirement for a balance between E2f3a and E2f3b transcriptional activities to maintain the correct dosage of Sox2 in stem and progenitor cells.

Although E2f3a/b and p107 are highly expressed in neural precursor cells, these proteins become rapidly down-regulated as cells undergo differentiation. Uncovering other mechanisms by which long-term repression of *Sox2* is maintained as cells differentiate will therefore be important. Notably, a recent study has shown that the Cyclin-dependent kinase inhibitor (CKI) p27 is required for repression of *Sox2* during differentiation of pluripotent stem cells (Li et al., 2012). p27 is recruited to the *Sox2* SRR2 enhancer and functions in a complex together with p130 and E2f4 to silence *Sox2* expression during differentiation. As the pocket protein p130 is highly expressed in differentiated cells and plays a key role in long-term silencing of cell cycle genes, it may well play a crucial role in silencing *Sox2* in post-mitotic neurons. Another recent study demonstrated that the CKI p21 represses *Sox2* expression in adult NSCs (Marqués-Torrejón et al., 2013). In adult NSCs it has been shown that p21 represses *Sox2* through the SRR2 enhancer, and its loss results in excessive *Sox2* expression and precursor exhaustion. This exhaustion, however, is preceded by an initial expansion of the precursor pool (Kippin, 2005; Marqués-Torrejón et al., 2013). In E2f3a deficient embryonic and adult NSCs, we found that elevated levels of Sox2 over-expression lead to increased self-renewal; however, it is also possible that E2f3a deficient NSCs may exhaust with time, following extended passaging *in vitro* or with advanced animal age. It is also conceivable that E2f3 may participate in the recruitment of p21 to its SRR2 enhancer binding site. However, given that the E2f3 and p21 binding sites have been identified in distinct regulatory domains of the *Sox2* gene, and E2f-independent mechanisms of p21

recruitment to *Sox2* have been suggested (Marqués-Torrejón et al., 2013), it is likely that they regulate *Sox2* expression by distinct mechanisms. Examining these questions will be an important focus for future studies, and will contribute to our understanding of molecular events underlying *Sox2* gene silencing in differentiating cells.

Through our identification of *Sox2* as a functional target gene of the pRb/E2f pathway in neural precursors, we have linked the cell cycle machinery with pluripotent gene regulation in a biologically relevant context. E2f dependent regulation of *Sox2* has clear functional consequences in the developing brain, however we suggest that this may be a common feature of stem cell regulation and the pluripotent state, as both Sox2 and pRb/E2f proteins are expressed in diverse tissue specific, pluripotent, and cancer stem cell populations (Galderisi et al., 2006; Pevny and Nicolis, 2010; Arnold et al., 2011). In addition, the pRb binding proteins RBBP4 and RBBP9 have recently been implicated in regulation of the pluripotent state in human stem cells, and E2f motifs were identified in the promoters of key pluripotency factors, including *NANOG*, *POU5F1*, *FOXD3* and *SOX2* (O'Connor et al., 2011). ChIP based experiments have further demonstrated that E2fs are found at the promoters of a large number of pluripotency related genes (Chen et al., 2008; O'Connor et al., 2011), although direct functional consequences for these interactions have not previously been described. In conclusion, these studies point to the possibility that E2fs may regulate other pluripotency factors in addition to *Sox2*, and support the idea that E2f3 dependent *Sox2* regulation is a fundamental mechanism in tissue specific, tumourigenic and pluripotent stem cell populations.

EXPERIMENTAL PROCEDURES

Mouse models

Germline *E2f3a* and *E2f3b* null mice were originally generated by G. Leone and were maintained on an FVBN background (Chen et al., 2007; Tsai et al., 2008). Animal experiments were approved by the University of Ottawa's Animal Care Ethics Committee, which abides by the guidelines of the Canadian Council on Animal Care. E2f3-*flox*/E2f3a- mice were generated by crossing E2f3a-/- mice with E2f3-*flox/flox* animals maintained on an SV129 background. p107 deficient mice were generated as previously described (LeCouter et al., 1998). All Adult mice analyzed were 2 months of age or older.

Neural precursor cultures

Neural precursors were obtained by dissection of the ventral (GE) or dorsal (cortex) telencephalic tissue of developing embryos; neurosphere and *in vitro* differentiation assays were performed as previously described(Vanderluit et al., 2004), with exception for the lentivirus experiments. Here, neural precursors were plated at a density of 5 cells/ul 7 days post infection, and the number of regenerated neurospheres were counted after 6 days in culture. All neurosphere assays were performed on 4-7 independent samples, from at least 2 separate experiments.

Western blotting, Immunohistochemistry, Cell Counts, Primers and Antibodies

Details are described in Supplemental Experimental Procedures.

Statistical Analysis

All statistical comparisons in this study were performed using an unpaired two-tailed t test, with differences considered significant with a p value of <0.05 (*), ** $p<0.01$, *** $p<0.001$.

Lentiviral infections

shRNA Lentiviral expression constructs were obtained from Open Biosystems and include a scrambled control (Cat. #RHS4346), shSox2-1 (clone ID 153337) and shSox2-2 (153339) plasmids. Neurosphere cultures were infected with lentiviral particles at a multiplicity of infection (MOI) of 30. For Sox2 over-expression, the *Sox2* coding sequence was sub-cloned into the MCS of an IRES-GFP backbone, viruses were produced and neurospheres were infected at an MOI of 5. For Cre expression experiments, GFP or Cre coding sequences were sub-cloned into the NCS of a pWPXLD plasmid, and cells were infected at an MOI of 10.

Luciferase Reporter Assays

Reporter assays were performed in HEK-293T cells as previously described (Andrusiak et al., 2011). E2f consensus site mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit and primer design software from Stratagene.

Chromatin Immunoprecipitation

ChIP analysis was performed as previously described (Andrusiak et al., 2011) in proliferating neurospheres, except that immuno-complexes were captured using protein A Dynabeads. RT-PCR was used to quantify ChIP enrichment values. Each experiment was

performed on at least three independent samples. ChIP-on-chip experiments were performed as previously described (Liu et al., 2010).

Fear Conditioning Analysis

Details are described in Supplemental Experimental Procedures

ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA INVENTORY

We have included seven supplemental figures, as well as supplemental text including figure legends, experimental procedures and three references.

Supplemental Figure 2.1: Relates to Figure 2.1 in the main text. This figure demonstrates that both E2f3 isoforms are expressed in GE and cortical neural precursors at different developmental time points. It also illustrates that only the appropriate E2f3 isoform is absent in neural precursors from E2f3a and E2f3b mutants, and that other pRb and E2f family members are not abnormally expressed in E2f3a/b knock-outs, demonstrating the specificity of our phenotypes. This figure also demonstrates a reduced neuronal commitment *in vivo* in *E2f3a*$^{-/-}$ mice and an increased commitment to differentiation in *E2f3b*$^{-/-}$ mice using two additional measurements than what is shown in the main text.

Supplemental Figure 2.2: Relates to Figure 2.2 in the main text. This figure demonstrates a progressive increase in proliferating precursor numbers over a developmental time-course in the *E2f3a*$^{-/-}$ cortex, and a steady decrease in precursor numbers in the *E2f3b*$^{-/-}$ cortex, by both PH-H3 immuno-staining and BrdU incorporation and staining. Additionally, quantification of PH-H3 positive cells in the VZ and SVZ separately demonstrates that VZ precursors are already expanded at E14.5 in *E2f3a*$^{-/-}$ mice, and that the VZ precursor population is specifically affected in E2f3b knockouts.

Supplemental Figure 2.3: Relates to Figure 2.2 in the main text. This figure demonstrates that later born layer I-III neurons show a greater reduction in number than early born layer VI neurons in the post-natal day 1 *E2f3a*$^{-/-}$ cortex, suggesting that the reduction of neurogenesis in these animals worsens with time. Alternatively, neurons in the *E2f3b*$^{-/-}$ newborn cortex are similarly increased in all layers.

Supplemental Figure 2.4: Relates to Figure 2.2 in the main text. This figure shows that E2f3a is expressed in a subset of cells in the cortical proliferative zones (the GE (ventral cortex) and the VZ/SVZ surrounding the lateral ventricle). It also demonstrates that E2f3a co-labels with Sox2 in the GE and Pax6 in the dorsal cortex, and that its expression in the dorsal cortex is confined to BrdU^{+} S phase precursors. Additionally, it shows neurosphere assay data at an additional time-point (E17.5) than what is shown in the main text. Lastly, measurements of the plane of division relative to the apical ventricular surface of wild-type and *E2f3a*$^{-/-}$ mitotic cells reveal that E2f3a knock-outs have more NPCs displaying vertical divisions, and fewer with horizontal divisions.

Supplemental Figure 2.5: Relates to Figure 2.3 in the main text. This figure shows that virtually all GE precursors *in vivo* and *in vitro* express Sox2.

Supplemental Figure 2.6: Relates to Figure 2.5 in the main text. Flow cytometry based cell cycle analysis shows that the percentage of cells in each phase of the cell cycle is similar between E2f3a and E2f3b control and knockout neurosphere cultures.

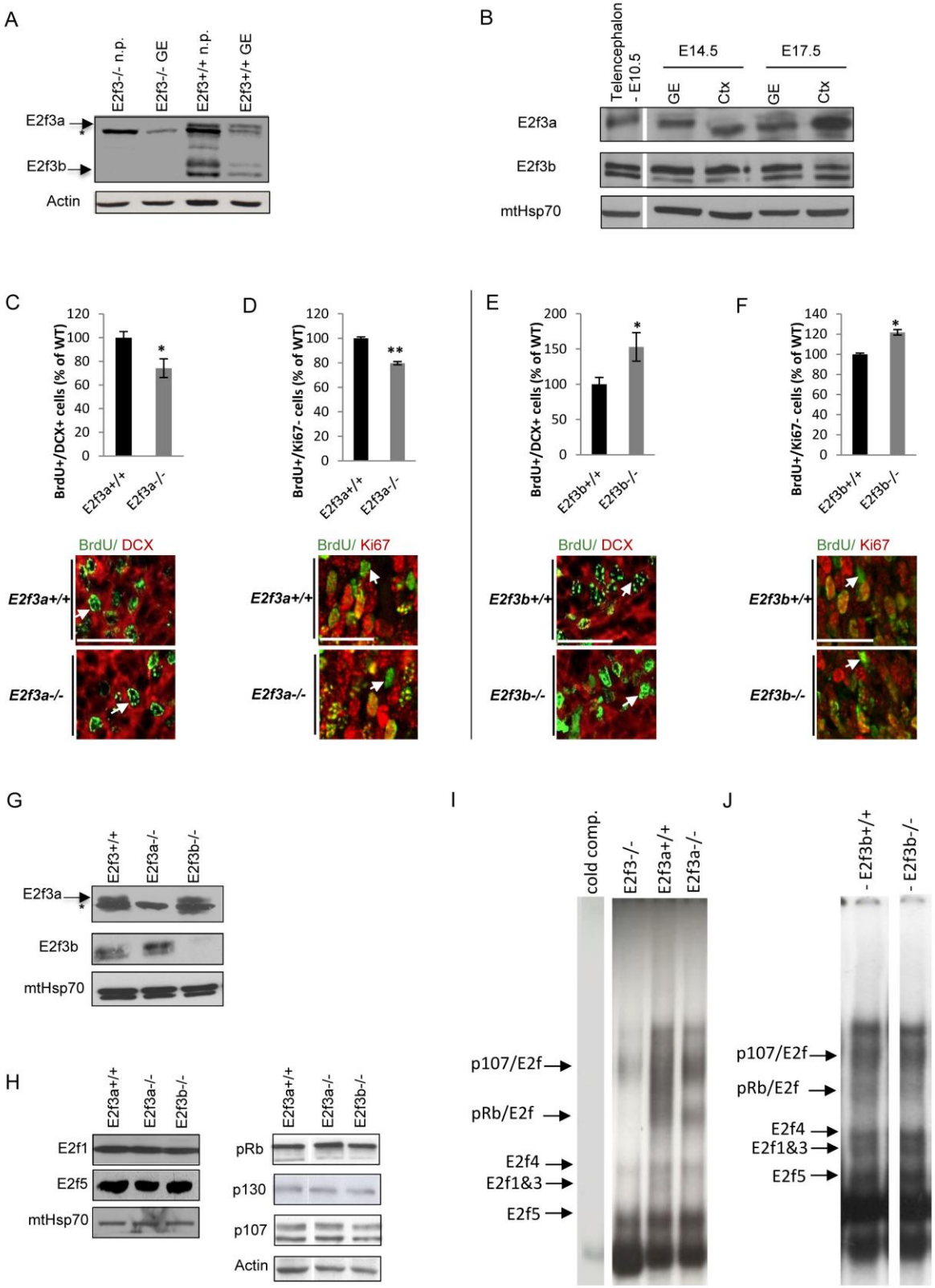
Supplemental Figure 2.7: Relates to Figure 2.6 in the main text. This figure includes control data for the fear conditioning studies, demonstrating that *E2f3a*$^{+/+}$ and *E2f3a*$^{-/-}$ mice are not different in their baseline freezing levels or in their threshold for response to the foot shock.

Cell Stem Cell, Volume *12*

Supplemental Information

Opposing Regulation of *Sox2* by Cell-Cycle Effectors E2f3a and E2f3b in Neural Stem Cells

Lisa M. Julian, Renaud Vandenbosch, Catherine A. Pakenham, Matthew G. Andrusiak, Angela P. Nguyen, Kelly A. McClellan, Devon S. Svoboda, Diane C. Lagace, David S. Park, Gustavo Leone, Alexandre Blais, and Ruth S. Slack


A
E2f3-/- n.p.
E2f3-/- GE
E2f3+/+ n.p.
E2f3+/+ GE
E2f3a
E2f3b
Actin
B
Telencephalon - E10.5
E14.5
E17.5
GE
Ctx
GE
Ctx
E2f3a
E2f3b
mtHsp70
C
BrdU+/DCX+ cells (% of WT)
E2f3a+/+
E2f3a-/-
BrdU/ DCX
E2f3a+/+
E2f3a-/-
D
BrdU+/Ki67- cells (% of WT)
E2f3a+/+
E2f3a-/-
BrdU/ Ki67
E2f3a+/+
E2f3a-/-
E
BrdU+/DCX+ cells (% of WT)
E2f3b+/+
E2f3b-/-
BrdU/ DCX
E2f3b+/+
E2f3b-/-
F
BrdU+/Ki67- cells (% of WT)
E2f3b+/+
E2f3b-/-
BrdU/ Ki67
E2f3b+/+
E2f3b-/-
G
E2f3+/+
E2f3a-/-
E2f3b-/-
E2f3a
E2f3b
mtHsp70
H
E2f3a+/+
E2f3a-/-
E2f3b-/-
E2f1
E2f5
mtHsp70
pRb
p130
p107
Actin
I
cold comp.
E2f3-/-
E2f3a+/+
E2f3a-/-
p107/E2f
pRb/E2f
E2f4
E2f1&3
E2f5
J
- E2f3b+/+
- E2f3b-/-
p107/E2f
pRb/E2f
E2f4
E2f1&3
E2f5

Supplemental Figure 2.1. Expression Pattern of pRb/E2f Family Members in Wild-Type and E2f3a- and E2f3b-Deficient Neural Precursors and Quantification of Newly Committed DCX$^+$ and Ki67$^-$ Cells In Vivo, Related to Figure 2.1

(A) Immunoblot for E2f3 in cultured neural precursors (n.p.) (neurospheres) and GE tissue at E14.5. Actin was used as a protein loading control. Arrows indicate the bands for E2f3a and E2f3b, * denotes a non-specific band.

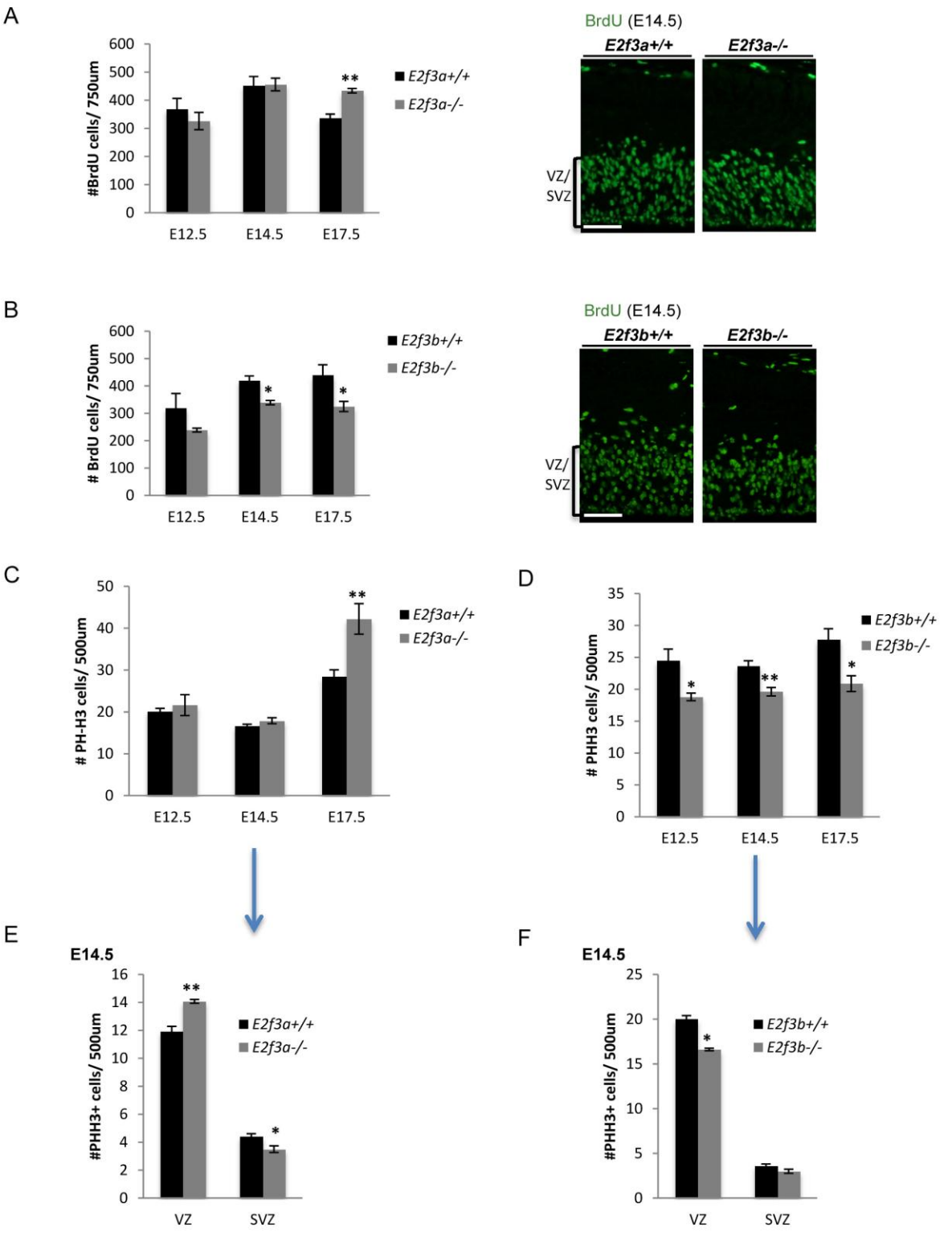
(B) Immunoblot for E2f3 in GE and dorsal cortex tissue (Ctx) at E14.5 and E17.5, and whole telencephalon at E10.5, demonstrating that both isoforms are expressed at different developmental stages and in telencephalic proliferative zones. mtHsp70 was included as a loading control.

(C-F) Pregnant mothers received an IP injection of BrdU at gestation day E13.5, embryos were sacrificed 24 hours later and their brains were sectioned coronally and processed for immuno-staining. The number of newly committed neurons in the dorsal cortex were identified as those cells expressing both BrdU and doublecortin (DCX), an immature neuronal marker. BrdU and DCX co-stained cells were quantified within a defined area through the SVZ and IZ of wild-type and knock-out E2f3a (C) and E2f3b (E) mice. Additionally, the number of newly committed cells that were no longer cycling were identified as BrdU$^+$ but Ki67$^-$ (D&F) (n=4). Results are presented as a percentage of the wild-type average +/- SEM (*$p<0.05$, **$p<0.01$).

(G) Immunoblot for E2f3 from E14.5 cultured neurospheres, demonstrating that expression of the other E2f3 isoform is not changed when the other isoform is absent. * non-specific band. mtHsp70 was included as a loading control.

(H) Immunoblot in neural precursor cultures from the indicated genotypes for pRb and E2f family members demonstrates a lack of compensatory expression changes in the absence of E2f3a or E2f3b. mtHsp70 and Actin were included as loading controls.

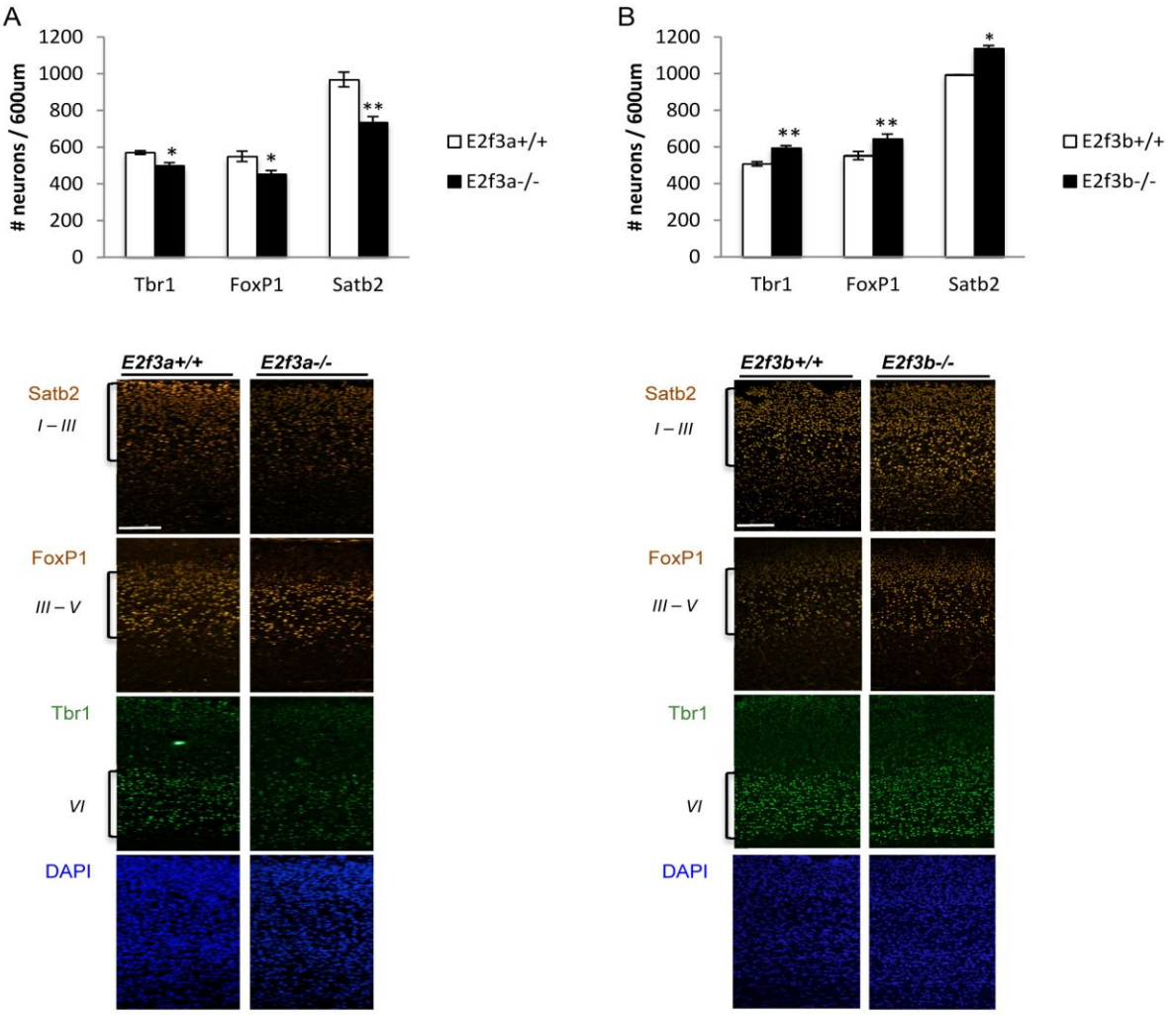
(I&J) Electrophoretic mobility shift assays demonstrate that DNA binding by other E2fs is not highly deregulated in the absence of E2f3a (I) or E2f3b (J).


A
#BrdU cells/ 750um
600
500
400
300
200
100
0
E12.5
E14.5
E17.5
**
E2f3a+/+
E2f3a-/-
BrdU (E14.5)
E2f3a+/+
E2f3a-/-
VZ/
SVZ
B
BrdU cells/ 750um
600
500
400
300
200
100
0
E12.5
E14.5
E17.5
*
*
E2f3b+/+
E2f3b-/-
BrdU (E14.5)
E2f3b+/+
E2f3b-/-
VZ/
SVZ
C
PH-H3 cells/ 500um
50
40
30
20
10
0
E12.5
E14.5
E17.5
**
E2f3a+/+
E2f3a-/-
D
PHH3 cells/ 500um
35
30
25
20
15
10
5
0
E12.5
E14.5
E17.5
*
**
*
E2f3b+/+
E2f3b-/-
E
E14.5
#PHH3+ cells/ 500um
16
14
12
10
8
6
4
2
0
VZ
SVZ
**
*
E2f3a+/+
E2f3a-/-
F
E14.5
#PHH3+ cells/ 500um
25
20
15
10
5
0
VZ
SVZ
*
E2f3b+/+
E2f3b-/-

Supplemental Figure 2.2. PH-H3 Immunostaining and 2 Hr BrdU Pulse in E2f3a and E2f3b Knockouts at Additional Time Points, Related to Figure 2.2

(A-D) Quantification of $BrdU^+$ (A&C) or $PH\text{-}H3^+$ (B&D) cells in the dorsal cortex following a 2hr BrdU pulse in E12.5, E14.5 and E17.5 sections from E2f3a (A&C) and E2f3b (B&D) wild-type and knockout mice. BrdU and PH-H3 expressing cells were quantified throughout both the VZ and SVZ.

(E&F) Quantification of the number of $PH\text{-}H3^+$ cells in the VZ and SVZ separately demonstrates that at E14.5 precursors in the VZ of *E2f3a*$^{-/-}$ mice are already expanded compared to *E2f3a*$^{+/+}$ (E), and that VZ precursors are the only population affected in *E2f3b*$^{-/-}$ mice (F). Results are presented as the mean number of cells per ventricular length of 500um (PH-H3) or 750um (BrdU) +/- SEM (*$p<0.05$, **$p<0.01$) (n=4). Scale bars = 100um.



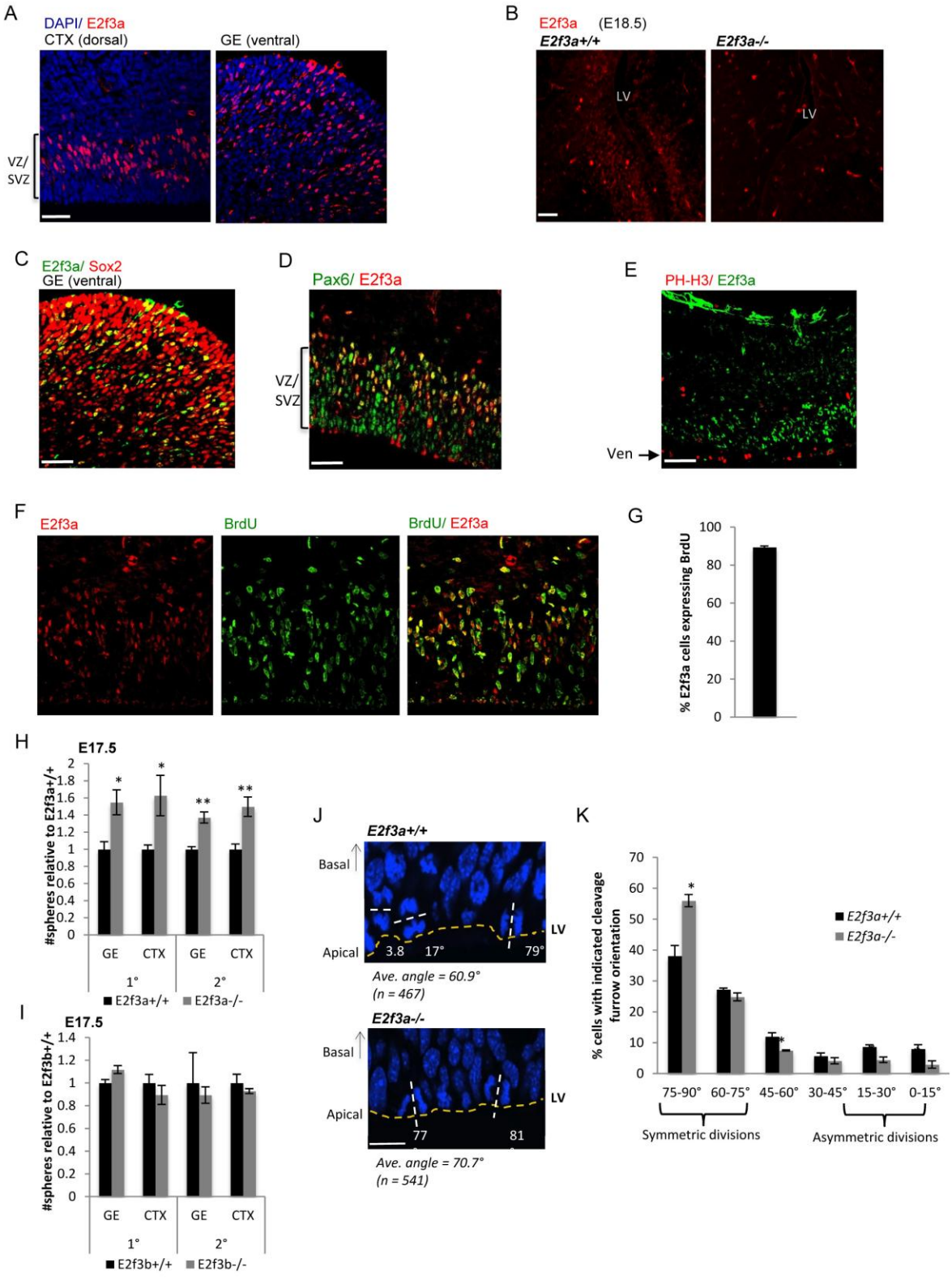
Supplemental Figure 2.3. Neuronal Output in *E2f3a*$^{-/-}$ and *E2f3b*$^{-/-}$ Newborn Cortex, Related to Figure 2.2

Brain sections at post-natal day 1 were stained for layer VI Tbr1^{+} neurons (early born), layer III-V FoxP1^{+} neurons, and layer I-III Satb2^{+} neurons (late born) to identify neuronal output in the newborn brain.

(A) In *E2f3a*$^{-/-}$ brains, the number of Tbr1^{+} neurons was decreased by 13%, FoxP1 by 18%, and Satb2 by 24, demonstrating that later born neurons are more reduced than early born neurons when E2f3a is absent (n=3).

(B) In *E2f3b*$^{-/-}$ brains, Tbr1^{+} neurons were increased by 15%, and FoxP1 and Satb2 were each increased by 13% (n=3).

Results are presented as the average number of positive neurons from 3 sections per animal over a 600um ventricular length +/- SEM. Scale bars = 50um.


A
DAPI/ E2f3a
CTX (dorsal)
GE (ventral)
VZ/
SVZ
B
E2f3a (E18.5)
E2f3a+/+
E2f3a-/-
LV
LV
C
E2f3a/ Sox2
GE (ventral)
D
Pax6/ E2f3a
VZ/
SVZ
E
PH-H3/ E2f3a
Ven
F
E2f3a
BrdU
BrdU/ E2f3a
G
% E2f3a cells expressing BrdU
100
80
60
40
20
0
H
E17.5
#spheres relative to E2f3a+/+
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
**
**
GE
CTX
GE
CTX
1°
2°
E2f3a+/+
E2f3a-/-
I
E17.5
#spheres relative to E2f3b+/+
1.4
1.2
1
0.8
0.6
0.4
0.2
0
GE
CTX
GE
CTX
1°
2°
E2f3b+/+
E2f3b-/-
J
E2f3a+/+
Basal
Apical
LV
3.8
17°
79°
Ave. angle = 60.9°
(n = 467)
E2f3a-/-
Basal
Apical
LV
77
81
Ave. angle = 70.7°
(n = 541)
K
% cells with indicated cleavage furrow orientation
70
60
50
40
30
20
10
0
*
*
E2f3a+/+
E2f3a-/-
75-90°
60-75°
45-60°
30-45°
15-30°
0-15°
Symmetric divisions
Asymmetric divisions

Supplemental Figure 2.4. E2f3a Expression in S Phase APs In Vivo, Analysis of Self-Renewal Defects by Neurosphere Assay at E17.5, and Mitotic Spindle Pole Analysis, Related to Figure 2.2

(A) Immunohistochemistry (IHC) using an antibody against the N-terminus of E2f3 (red) at E14.5 demonstrates expression of E2f3a in a subset of cells in the VZ/SVZ within the dorsal cortex, and the GE.

(B) IHC for E2f3a at E18.5 in wild-type and E2f3a deficient brain sections demonstrates specificity of E2f3a detection around the lateral ventricle (LV) by our antibody.

(C) IHC for E2f3a (green) and Sox2 (red) in the GE at E14.5 shows that E2f3a is also expressed in ventral telencephalic $Sox2^{+}$ precursors.

(D) Pax6 was used as an independent marker of apical precursor cells. IHC demonstrates expression of E2f3a in a subset of $Pax6^{+}$ cells in the dorsal cortex.

(E) IHC for E2f3a and PH-H3 in the E14.5 dorsal cortex, indicates no expression of E2f3a in mitotic cells (Ven = ventricle).

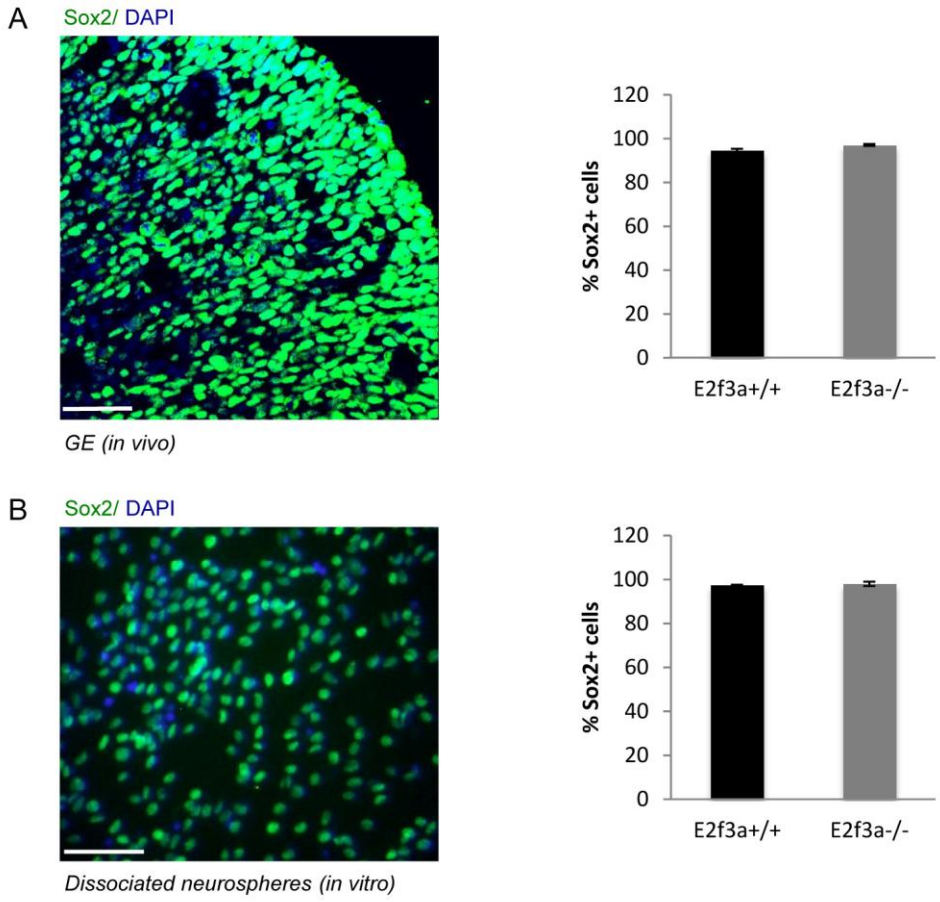
(F) Brain sections that received a 2hr BrdU pulse were stained for BrdU and E2f3a, and were imaged along the dorsal cortex.

(G) Quantification of the number of E2f3a expressing cells (per section) that also express BrdU following a 2hr BrdU pulse. The majority (89%) of $E2f3a^{+}$ cells express BrdU and are therefore in S phase. Results are presented as averages +/- SEM (n=3). Scale bars for A-F is 100um.

(H&I) Primary and secondary neurosphere assays were performed at E17.5 from wild-type and knockout E2f3a (H) and E2f3b (I) neurosphere cultures derived from both the GE and dorsal cortex (Ctx). Again, as with E14.5 neurosphere assays, E2f3a deficiency leads to increased neurosphere numbers and self-renewal, while E2f3b loss has no effect. The data is presented as mean +/- SEM (n=4).

(J) Orientation of the mitotic spindle pole has been linked to asymmetric versus symmetric divisions due to the segregation pattern of cell fate determinants. Vertical cleavage planes (60-90°) are associated with symmetric divisions, and horizontal (0-30°) and intermediate (30-60°) cleavage planes correlate with asymmetric divisions. Chromatin was visualized by DAPI, and lines were drawn to trace the apical surface of the lateral ventricle (LV) (yellow line) or through sister chromatids to mark the cleavage furrow (white line). The smallest angle between them was calculated. Scale bar = 10um.

(E) Spindle pole angle measurements were collected for over 460 cells per genotype from 3 separate animals. The percentage of all cells measured that fell within specific angle ranges was calculated. E2f3a loss results in a greater percentage of vertical cleavage planes (associated with symmetric divisions), and a lower percentage of horizontal cleavage planes (correlated with asymmetric divisions). Mean values +/ SEM is shown.

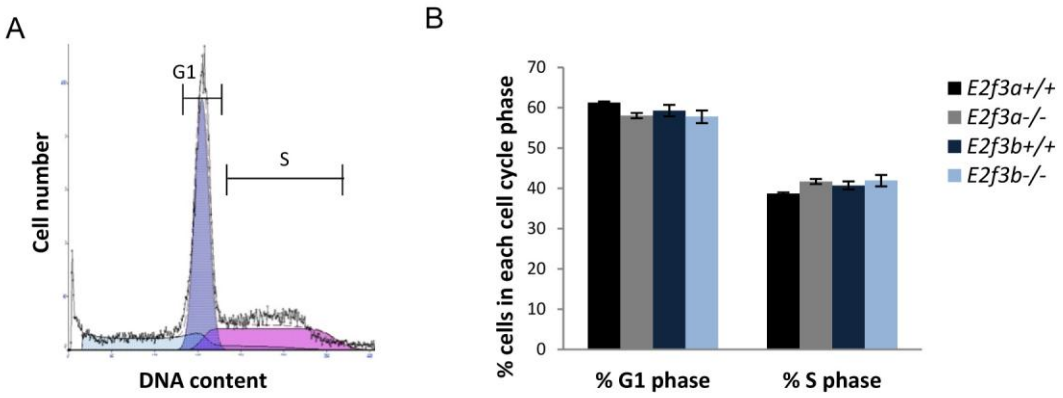


Supplemental Figure 2.5. Almost all GE Precursors In Vivo and In Vitro Express Sox2, Related to Figure 2.3

(A) E14.5 brain sections were stained for DAPI and Sox2 and the ganglionic eminence (GE) was imaged. The structure shown in the image is representative of the GE tissue that is obtained in our dissections and that was used in our immunoblot analysis. Quantification of the percentage of $DAPI^{+}$ cells that are also $Sox2^{+}$ shows that 95% of *E2f3a*$^{+/+}$ and 97% of *E2f3a*$^{-/-}$ GE cells *in vivo* express Sox2, and there is no significant difference between genotypes. Results are presented as the mean +/- SEM for n=4. Scale bar is 100um.

(B) In neurosphere cultures derived from E14.5 GE dissections, 97% and 98% of cells from *E2f3a*$^{+/+}$ and *E2f3a*$^{-/-}$ cultures, respectively, express Sox2.

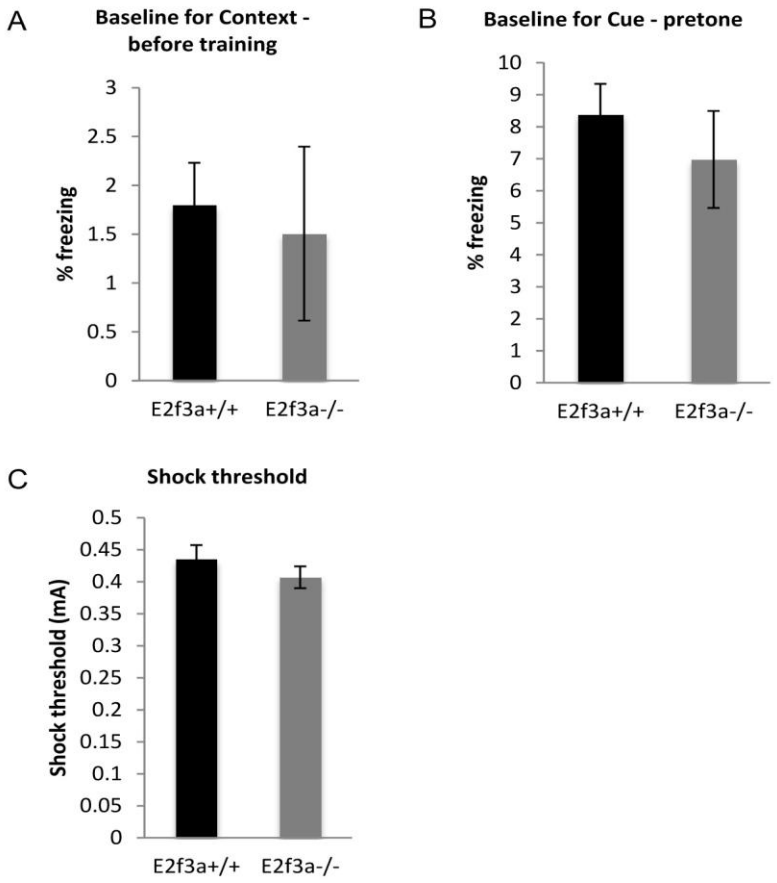
Results are presented as the mean, and error bars representing the range of values, for n=2. Scale bar is 100um



Supplemental Figure 2.6. Loss of E2f3 Isoforms Does Not Alter Cell-Cycle Dynamics, Related to Figure 2.5

(A) Actively proliferating neurosphere cultures were fixed, stained with DAPI and prepared for flow cytometry. The number of cells in G1 and S phases of the cell cycle was determined based on 2N and 4N DNA, respectively, as determined by DAPI fluorescence.

(B) The percentage of cells in G1 or S phases was similar in E2f3a and E2f3b control and knockout neural precursors. Data shown is the mean +/- SEM (n=4).



Supplemental Figure 2.7. Baseline Freezing For Context and Cue Experiments and Shock Threshold are Unchanged Between Genotypes, Related to Figure 2.6

(A) Mice were placed in the fear conditioning apparatus for two minutes before the first foot shock was administered, and the percent of time spent freezing was measured and reported as baseline freezing relevant to the context. *E2f3a*$^{+/+}$ and *E2f3a*$^{-/-}$ mice exhibit similar baseline freezing levels, and baseline levels were significantly lower than post-training contextual freezing levels (data not shown) (*E2f3a*$^{+/+}$: $p=4.06x10^{-9}$; 24-fold decrease).
(B) Following fear conditioning training and prior to cue testing, mice are placed in a second novel environment and the percent of time spent freezing during a 3 minute duration was recorded. This represents the pre-tone or baseline for the cue testing; *E2f3a*$^{+/+}$ and *E2f3a*$^{-/-}$ mice exhibit similar freezing levels, and baseline levels were significantly lower than post-tone cue testing freezing levels (data not shown) (*E2f3a*$^{+/+}$: $p=1.39x10^{-18}$;8.4-fold decrease). Data for panels A&B is presented as mean +/- SEM (n=18 for *E2f3a*$^{+/+}$, n=13 for *E2f3a*$^{-/-}$).
(C) *E2f3a*$^{+/+}$ and *E2f3a*$^{-/-}$ mice were exposed to gradually increasing levels of foot shock intensity to determine their relative shock thresholds. Shock threshold was identified when the mouse exhibited vocalization concurrent with movement two consecutive times at the same shock level. There is no difference in shock threshold between genotypes. Data is presented as mean +/- SEM (n=10 for *E2f3a*$^{+/+}$, n=7 for *E2f3a*$^{-/-}$).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Immunohistochemistry and Western blotting

For immunohistochemistry, tissue was treated and sectioned as previously described (Mcclellan et al., 2007). Before antibody incubation, antigen retrieval was performed by heating sections at 95°C for 30 minutes in DAKO retrieval solution, followed by 25 minutes at room temperature. For BrdU incorporation, pregnant mice were given a single IP injection of 50mg/kg BrdU and were harvested 2 hours later to visualize cells in S phase, or 24 hours later for commitment assays. Prior to immunostaining, antigen retrieval was performed followed by incubation in 2N HCl at 37°C for 30 minutes and neutralization in 0.1M Na borate, pH8.5, for 15 minutes at room temperature. Protein isolation and Western blots were performed as previously described (Andrusiak et al., 2011). Additionally, lysed samples were sonicated for 3 x 10 seconds at 25% intensity, and debris was spun down. Quantification was performed using ImageJ software and at least 3 independent sets of control and knockout material were included for these experiments.

Cell counts, Measurements

For PH-H3 and short term BrdU counts, brightly labeled cells in the VZ and SVZ were quantified along a ventricular length of 500 or 750um. The number of E2f3a^{+} cells that also express Sox2, Pax6, Tbr2 or BrdU were quantified in the dorsal cortex along a ventricular length of 500um. Sox2 and Tbr2 were quantified along the entire dorsal cortex, and cortical layer markers were counted over a ventricular length of 600um. For commitment assays, co-labeled cells (or BrdU+/Ki67-) were quantified along a ventricular length of 375um,

throughout the width of the SVZ and IZ. NeuroD1 and DCX were quantified in every 12th (40um) section throughout the rostral caudal length of the DG, and the number of positive cells from all sections was multiplied by 12 to obtain the estimated cell number per DG. For SVZ counts, every 6th coronal section (14 um) from the most rostral crossing of the corpus callosum to the third ventricle (crossing of the anterior commissure) was stained for DCX. The DCX^{+} area was measured using ImageJ, and the area from all sections combined was multiplied by 6 to obtain the estimated DCX^{+} area per SVZ.

Antibodies

Antibodies against the following proteins were used in this study: PH-H3 (Millipore, 06-570), BrdU (Accurate Chemicals, OBT0030), DCX (Santa Cruz, sc-8066), βIII-tubulin (Tuj1) (J L Vanderluit et al., 2007), E2f3a (NeoMarkers, MS-1063-P1), Sox2 (sc-17320), Tbr2 (Abcam, AB-23345), Pax6 (Covance, PRB278P) and Ki67 (Cell Marque, SP6), E2f3 (pan-E2f3) (sc-878), p107 (sc-318), H3K4Me3 (Millipore, CS200580), H3K27Me3 (ABE44), RNA Polymerase II (Covance, MMS-126R), mtHsp70 (Thermo Scientific, MA3-028), E2f1(sc-193), E2f5 (sc-1083x), pRb (BD, 554136), p130 (sc-317), and NeuroD (sc-1086).

Primers

Primers used for ChIP analysis on the *Sox2* promoter:

Upstream Forward primer: 5'-CAGAAACAATGGCACACCAC-3'

Upstream Reverse primer: 5'-CAAGACGACAGCTCCTTTCC-3'

TSS Forward primer: 5'-CCCATTTATTCCCTGACAGC-3'

TSS Reverse primer: 5'-CTCTTCTTTCTCCCAGCCCTA-3'

Primers for amplification of the *Sox2* promoter fragment (-800bp to +285bp):

Forward primer: 5'-GCCTTTGCACCCTTTGGATGG-3'

Reverse primer: 5'-CGCGGAGATCTGGCGGAGAA-3'

Primers for Poly-A mutagenesis of E2f consensus motif in *Sox2* promoter fragment:

Forward primer: 5'-TTGCCCCACCCTGGCCCCAGCTAAAAACGCCCCATCCACC-3'

Reverse primer: 5'-GGTGGATGGGGCGTTTTTAGCTGGGGCCAGGGTGGGGCAA-3'

Mitotic Spindle Pole Analysis

To quantify the orientation of the mitotic spindle pole, E14.5 cortical sections were stained with DAPI to visualize chromatin and cells undergoing anaphase were imaged along the apical surface of the lateral ventricle. Using ImageJ, one line was drawn to trace the surface of ventricle and a second line traced the cleavage furrow, bisecting the spindle poles. The smallest angle formed between the two intersecting lines was calculated. At least 460 anaphase cells were measured for each genotype, and the final data combines measurements from 5 distinct rostral-caudal levels in 3 different animals. Protocol adapted from (Godin et al., 2010).

Fear Conditioning Analysis

Fear conditioning experiments were performed according to (Villasana et al., 2009), with few modifications. Briefly, on training day (day 1) mice were placed in an Ethovision PhenoTyper clear box (Noldus Information Technology, North America) for 2 minutes, and were given a 30 second, 90 dB tone co-terminating in a 2 second, 0.45 mA foot-shock

delivered twice with a 1 minute inter-stimulus interval. On day 2 (~24 hours after training), the contextually conditioned fear testing was performed by placing the mice back into the same fear conditioning apparatus (context) for a total duration of 6 minutes. On day 3 (~48 hours after training), cue conditioned fear testing was performed to measure the amount of freezing in a new context, as well as freezing occurring in the new context with a tone that was previously paired with a foot shock during training. The new context consisted of the same type of apparatus, however the mice were placed into a different box than the one they were trained in, and the conditions for testing were modified by changing the interior shape of the apparatus, removing white noise from the testing room, adding the odor of vanilla, changing lighting conditions, changing floor and wall texture, and using a different handling technique to place mice into the box. For cue testing, the mice were allowed to freely explore for 3 minutes before re-exposure to the fear conditioning tone for a duration of 3 minutes. During training and context and cue testing, the duration and percent of time spent freezing (immobile) was recorded using Ethovision 8 XT video tracking system as previously described (Pham et al., 2009). To exclude potential effects of anxiety-like or locomotor behavior contributing to differences in freezing behavior, immobility was also analyzed during the first 2 minutes of fear conditioning training occurring on Day 1 prior to the tone/shock exposure, as well as during cue testing on Day 3 prior to tone exposure. Statistical analysis was performed using SPSS Version 19 and outliers were removed prior to analysis comparing *E2f3a*$^{+/+}$ (n=18) and *E2f3a*$^{-/-}$ (n=13) animals using a two-tailed unpaired t-test (*$p<0.05$).

Foot Shock Sensitivity Analysis

Shock sensitivity/ threshold analysis was performed as previously described (Klemenhagen et al., 2005). Briefly, mice were placed into the fear conditioning apparatus for 2 minutes followed by a 1 second foot shock at 0.05mA which was gradually increased in intensity by 0.05mAs. Mice received 2 exposures to each shock level with a random inter-stimulus interval ranging from 30 to 60 seconds. The intensity required for each mouse to flinch, move, jump and vocalize was recorded by an observer (who was blind to genotype) and the test was stopped when shock threshold was achieved as indicated by the mouse moving accompanied by either jumping or vocalization in response to the same shock intensity for two consecutive exposures. Statistical analysis was performed using SPSS Version 19 and outliers were removed prior to analysis comparing *E2f3a*$^{+/+}$ (n=10) and *E2f3a*$^{-/-}$ (n=7) animals using a two-tailed unpaired t-test (*$p<0.05$).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays (EMSAs) were performed on total protein extracts from *E2f3a*$^{-/-}$, *E2f3b*$^{-/-}$, or wild-type control cultured neural precursor cells, as previously described (Mcclellan et al., 2007).

Flow Cytometry

Proliferating neurosphere cultures were harvested, triturated, fixed in 1%PFA for 1hr at 4C, and in 70% EtOH at 4C overnight. Cell pellets were washed with 1XPBS and resuspended in 1ug/ml DAPI for 30 minutes at room temperature. Cells were analyzed for DNA content using a Dako MoFlo Legacy Flow Cytometer (Beckman Coulter) and Dako Summit Software v4.3. Cell cycle analysis was performed using MultiCycle analysis software.

CHAPTER 3

Lisa M Julian, Yubing Liu, Catherine A Pakenham, Gustavo Leone, Ruth S Slack[*], and Alexandre Blais[*]. (2013). Tissue-specific Binding of E2f3 isoforms to Promoters of Diverse Cell Fate Regulatory Genes in Neural Precursor Cells. *In preparation*

[*]*Indicates co-corresponding authors*

The majority of experiments and analyses were performed by LMJ, with assistance from AB. AB performed the boxplot, expression clustering and DNA motif enrichment analyses. YL and CAP provided technical training and support. AB and RSS provided technical training, conceptual insight and editorial support. GL provided knockout mouse colonies. The manuscript was written by LMJ, with critical review by AB and RSS.

Tissue-specific Binding of E2f3 Isoforms to Promoters of Diverse Cell Fate Regulatory Genes in Neural Precursor Cells

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ABSTRACT

The E2f transcription factors have emerged as important regulators of neural precursor cell (NPC) fate decisions in the developing and adult brain; an appreciation of the full extent of E2f functions in NPCs has been precluded, however, by potential functional redundancy among family members. We conducted a series of ChIP-chip experiments to identify promoters and associated target genes bound by E2f3 isoforms (E2f3a and E2f3b) and E2f4 in proliferating NPCs, to determine the potential of E2f function in the brain. We uncovered an extensive number of E2f bound gene promoters with key roles in controlling diverse cell fate regulatory processes including not only cell proliferation, but also self-renewal, cell death, migration and neurogenesis. The majority of targets are bound by multiple E2fs at transcriptionally active promoters, suggesting significant functional redundancy and a much more expansive role for E2fs in NPCs than previously appreciated. We also observed extensive tissue-specificity in gene expression and target gene binding for E2f3 isoforms, a feature not previously observed for E2f proteins, where 85% and 70% of E2f3-bound peaks in NPCs and myoblasts are specific to that tissue type. Our data reveal that classical cell cycle regulatory processes are conserved, but developmental and differentiation-related processes are remarkably tissue-specific, among E2f3 targets. Finally, we demonstrate a striking enrichment of the CTCF transcription factor at E2f3 bound sites, predominantly in NPCs and at the promoters of genes involved in regulating nervous system-dependent processes, suggesting a potential regulatory co-factor for E2f3 in controlling tissue-specific functions in neural precursors. Our findings demonstrate a wide spread role for E2fs in diverse NPC functions, and reveal the unexpected finding that E2f3 isoforms target gene promoters in a highly tissue-specific manner.

INTRODUCTION

The pRb/E2f pathway is a well-established regulator of proliferation, cell cycle exit and apoptosis; however, findings from *in vivo* knock-out models and analysis of E2f target genes over the past several years have revealed more diverse functions for this pathway. These include regulation of genes that control DNA damage repair, chromatin organization, cellular metabolism, and development and differentiation related processes (Muller, 2001; Ren, 2002; McClellan and Slack, 2007; Blanchet et al., 2011), a fundamental role in the regulation of diverse cell fates. This is particularly evident in the developing and adult nervous system, where roles in controlling precursor self-renewal, proliferation, asymmetric versus symmetric cell division, neuronal commitment, cell death, and migration have been observed for the pRb/E2f pathway in knock-out models (Cooperkuhn et al., 2002; Vanderluit et al., 2004; Ferguson et al., 2005; Chen et al., 2007; Mcclellan et al., 2007; Ruzhynsky et al., 2007; Vanderluit et al., 2007b; Mcclellan et al., 2009; Julian et al., 2013). These studies have suggested specific functions for individual family members, particularly among E2fs: E2f3 regulates neuronal migration, loss of E2f1 or E2f3b inhibits neural precursor cell proliferation, E2f3a loss enhances while E2f4 loss inhibits NPC self-renewal, and E2f3a and E2f3b antagonistically control neurogenesis (Cooperkuhn et al., 2002; Mcclellan et al., 2007; Ruzhynsky et al., 2007; Mcclellan et al., 2009; Julian et al., 2013).

Given these unique functions, a number of target genes and regulatory pathways under E2f control have been discovered that are distinct from canonical E2f cell cycle regulatory functions. These include genes involved in the Notch/Hes (Vanderluit et al., 2004), Fibroblast Growth Factor (Fgf) (Mcclellan et al., 2009) and Sonic Hedgehog (Shh) pathways (Ruzhynsky et al., 2007), and the neurogenesis and migration related genes

Dlx1/Dxl2 and *Neogenin* (Andrusiak et al., 2011; Ghanem et al., 2012), as well as the pluripotency factor *Sox2* (Julian et al., 2013). In these cases, deregulation of gene expression is observed with the loss of a single E2f protein, and associated biological phenotypes suggest that at least some target genes may be regulated by specific E2f family members or in a differential, antagonistic manner by more than one E2f. Previous studies that have identified E2f bound target genes on a broad scale have indicated, however, that E2fs predominantly bind at overlapping sites, both within a given cell type and between multiple tissues (Xu et al., 2007). These findings, combined with the generally mild phenotypes of individual E2f knock-outs, suggest extensive functional redundancy between E2fs. Thus, it is likely that E2fs regulate many more target genes and perhaps unique cellular functions in brain tissues than we have been able to appreciate with current animal models.

An understanding of the full potential of E2f function necessitates an appreciation of the repertoire of E2f target genes. Large-scale ChIP-chip and ChIP-Seq analyses of E2f1 and E2f4 targets have been reported in a number of immortalized and transformed cell lines (Conboy et al., 2007; Xu et al., 2007; Lee et al., 2011), and targets for the E2f3a and E2f3b isoforms have been reported only in C2C12 myoblasts (Asp et al., 2009). These studies have revealed largely overlapping functions among E2fs, with the exception of E2f3, where a unique preference for E2f3a and E2f3b in the regulation of cell cycle and differentiation related genes, respectively, has been demonstrated in muscle (Asp et al., 2009). Alternatively, E2f3 isoforms exhibit redundant functional roles in the context of mouse development (Tsai et al., 2008). Contrary to this finding, we have reported opposing roles, as well as antagonistic transcriptional activities at the *Sox2* locus, for E2f3a and E2f3b in the developing brain (Julian et al., 2013), suggesting that they may also bind unique target genes

in neural tissue. E2f target genes have not been evaluated in neuronal tissue on a genome-wide basis, and an analysis of E2f targets in primary cells in general is currently lacking. While the E2f family is composed of eight family members, E2f3 (including both E2f3a and E2f3b isoforms) and E2f4 have emerged as particularly important regulators of cell fate processes within the brain, and are highly expressed in both dorsal and ventral embryonic and adult NPC populations (Ferguson et al., 2002; Mcclellan et al., 2007; Ruzhynsky et al., 2007; Julian et al., 2013). Thus, an analysis of E2f3 and E2f4 dependent target genes in NPCs is essential to appreciate how E2fs may regulate diverse cell fates in the brain, and will also determine if E2f3 isoforms exhibit common and/or tissue specific target genes.

In order to test our hypothesis that E2fs target a wide range of genes involved in NPC fate decisions, and to determine if E2f family members exhibit unique genomic binding patterns in neural tissue, we used a combination of bioinformatic approaches to assess E2f3a, E2f3b and E2f4 target genes and their functional relevance in self-renewing neural precursor cells. We present evidence suggesting that E2fs are pervasive regulators of diverse NPC cell fate processes, and that they co-operate to control the expression of target genes in this context, as opposed to regulating distinct classes of genes. Stemming from these findings, we further reveal the surprising result that E2f3 exhibits extensive tissue-specificity in target gene binding in neural and muscle precursors, providing the first example of an E2f factor with predominantly distinct genomic binding patterns in different proliferating populations.

RESULTS

Identification of E2f promoter occupancy in neural precursors

To understand the potential breadth of E2f function in neural precursor cells (NPCs), we identified genomic binding sites for E2f3 and E2f4 in neurosphere cultures derived from telencephalic tissue at embryonic day 14.5 (E14.5), a peak stage of cortical neurogenesis. We determined the promoter occupancy of E2fs in proliferating NPCs by coupling chromatin immunoprecipitation (ChIP) using antibodies towards E2f3 and E2f4 with proximal promoter DNA microarrays (ChIP-chip). These antibodies have been successfully used previously in ChIP-based experiments (Xu et al., 2007; Asp et al., 2009; Julian et al., 2013), and we show here that they result in significant protein enrichment (relative to IgG) at known E2f target promoters (Thymidine Kinase (*TK1*) and *p107*), but not at the *Chrna1* promoter, which serves as a negative control (Blais, 2005) (Fig. 3.1A&B). In a set of pilot ChIP-chip experiments using a DNA microarray on which the full sequence of mouse chromosome 7 was tiled at an average resolution of one probe every 250bp, we estimated that 89% of E2f3 and 85% of E2f4 genomic binding sites are located between 3.5kb upstream and 1.5kb downstream of a transcriptional start site (TSS) (Supplemental Fig. 3.1A&B). The remaining 11% and 15% of E2f binding peaks are estimated to lie more than 10kb upstream or 3.5kb downstream of the nearest TSS. In light of these results, we designed tiling arrays containing DNA probes spanning 5kb upstream to 3kb downstream of the TSS of approximately 28,000 well curated mouse transcripts, as defined by the RefSeq database. This strategy gave us extensive

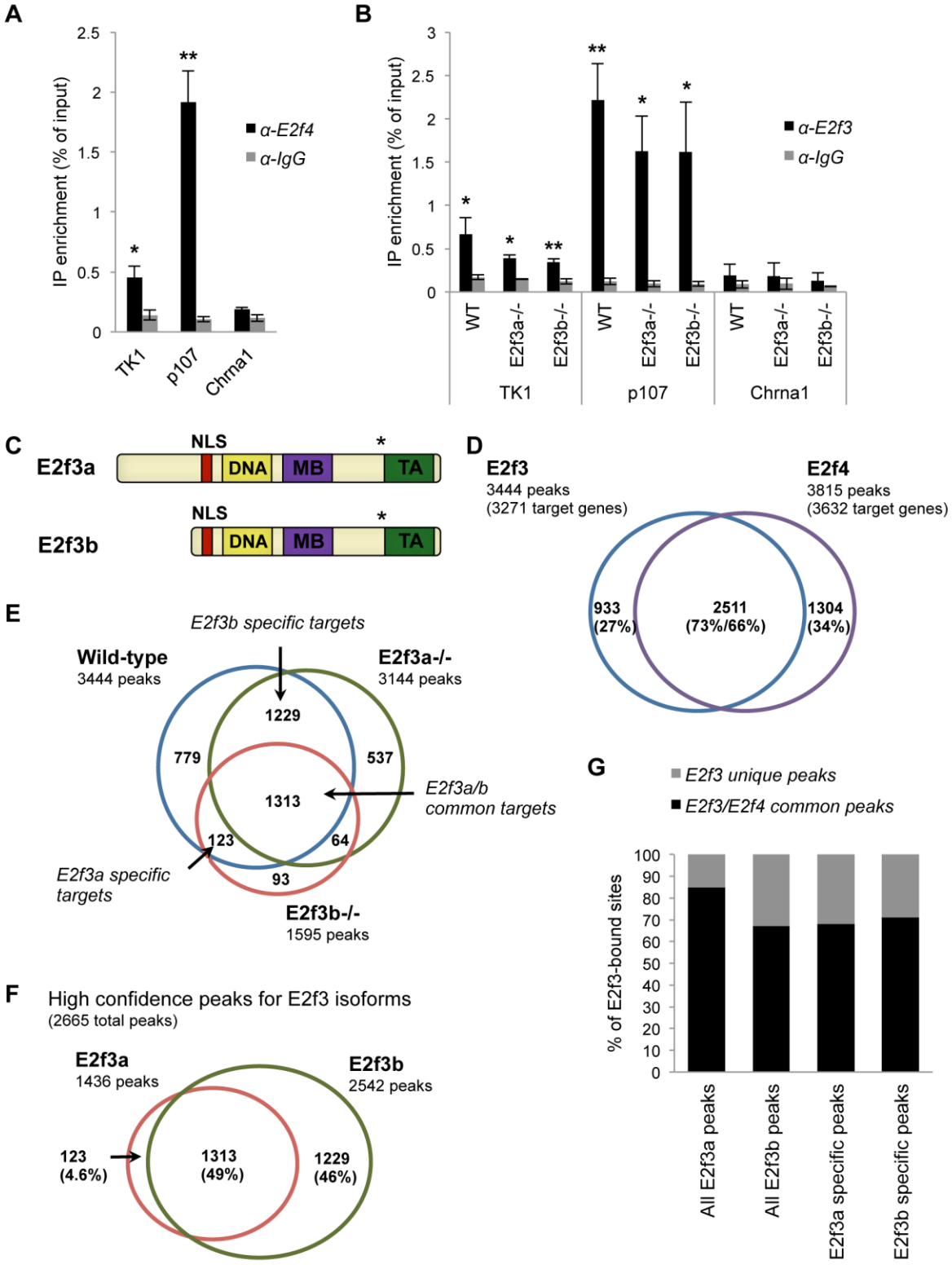

A
IP enrichment (% of input)
α-E2f4
α-IgG
TK1
p107
Chrna1
B
IP enrichment (% of input)
α-E2f3
α-IgG
WT
E2f3a-/-
E2f3b-/-
TK1
p107
Chrna1
C
E2f3a
E2f3b
NLS
DNA
MB
TA
D
E2f3
3444 peaks
(3271 target genes)
E2f4
3815 peaks
(3632 target genes)
933
(27%)
2511
(73%/66%)
1304
(34%)
E
E2f3b specific targets
Wild-type
3444 peaks
E2f3a-/-
3144 peaks
1229
779
537
1313
E2f3a/b
common targets
123
64
93
E2f3a specific
targets
E2f3b-/-
1595 peaks
F
High confidence peaks for E2f3 isoforms
(2665 total peaks)
E2f3a
1436 peaks
E2f3b
2542 peaks
123
(4.6%)
1313
(49%)
1229
(46%)
G
E2f3 unique peaks
E2f3/E2f4 common peaks
% of E2f3-bound sites
All E2f3a peaks
All E2f3b peaks
E2f3a specific peaks
E2f3b specific peaks

Figure 3.1. Specificity and redundancy among E2f3 and E2f4 genomic binding sites in neural precursor cells

(A) Quantitative ChIP (qChIP) analysis of E2f4 and IgG experiments to assess binding at the promoters of previously identified E2f targets (*thymidine kinase* (*TK1*) and *p107*) and a negative control gene (*Chrna1*) in WT neurospheres. E2f4 is significantly enriched over rabbit IgG at positive control genes only. (*$p<0.05$, **$p<0.01$). Graphed are the averages of 4-6 biological replicates, +/- SEM.

(B) qChIP analysis to assess E2f3 and rabbit IgG binding at the *TK1*, *p107* and *Chrna1* promoters in WT, E2f3a-/- and E2f3b-/- neurospheres. E2f3 binds to positive control promoters only, with similar enrichment in WT and knock-out cells. (*$p<0.05$, **$p<0.01$). Graphed are the averages of 3-4 biological replicates, +/- SEM.

(C) Schematic diagram of E2f3a and E2f3b protein structure. Both isoforms share common structural domains: DNA = DNA binding domain; MB = marked box domain; NLS = nuclear localization sequence; TA = transcriptional activation domain. The region recognized by the α-E2f3 antibody is denoted by *.

(D) Venn diagram demonstrating the degree of overlap between E2f3 and E2f4 peaks discovered by ChIP-chip. The number of peaks in each category is shown, with the percent of all E2f3 or E2f4 peaks in that category in parentheses.

(E) Venn diagram demonstrating the overlap between E2f3 peaks identified by ChIP-chip in WT, E2f3a-/- and E2f3b-/- neurospheres. Peaks identified as specific or common to E2f3a and E2f3b are indicated.

(F) Venn diagram demonstrating the peaks identified as common or unique for E2f3 isoforms. E2f3a binds a subset of E2f3b bound sites.

(G) Analysis of the percent of E2f3 peaks in the indicated categories that overlap with E2f4 peaks in NPCs.

coverage of promoters and neighbouring sequences, and allowed us to identify the location of E2f3&4 binding sites at virtually all known promoter regions in the mouse genome.

Previous studies have shown that the majority of E2f binding sites are within close proximity of a TSS, many belonging to genes that are functionally validated transcriptional targets of E2fs (Ren, 2002; Xu et al., 2007; Lee et al., 2011). Thus, for each E2f binding peak identified, we assigned a single target gene based on the gene whose TSS is closest to the peak. Additionally, to ensure that high stringency binding sites are reported, the results shown for each E2f protein are the average of three biologically independent replicate experiments, where a particular peak must be enriched in at least two separate experiments to be considered a valid binding event.

Specificity and redundancy among E2f3 and E2f4 target promoters

We first identified E2f3 binding sites in wild-type (WT) cells using an antibody that binds to a C-terminal epitope of the protein, which recognizes both E2f3 isoforms (Fig. 3.1C). We identified 3444 peaks enriched for E2f3 in WT NPCs, corresponding to 3271 unique target genes (Fig. 3.1D), at a false discovery rate (FDR) of less than 10%. This FDR cut-off has been used in a number of previous studies with a similar experimental protocol to successfully identify transcription factor binding sites for E2fs and other factors (Jin et al., 2006; Vokes et al., 2008; Liu et al., 2010). We observed an enrichment in the proportion of E2f3 peaks that contained an E2f consensus motif sequence compared to randomly selected control promoter regions, with the highest ranked peaks exhibiting the greatest motif enrichment (see below), suggesting that our analysis parameters have successfully identified E2f binding sites. We found slightly more binding sites for E2f4, with 3815 distinct peaks

and 3632 target genes (Fig 3.1D). In total, we identified 5459 unique E2f peaks in NPCs, and found that 28% of proximal promoter regions are bound by at least one E2f. Direct comparison of peaks enriched for E2f3 and E2f4 demonstrated that a large fraction of their binding sites overlap (73% or 66% of E2f3 or E2f4 targets, respectively) (Fig. 3.1D); however, approximately one third of the peaks enriched for each protein were unique, suggesting the existence of factor-specific target genes.

E2f3a binds to a subset of E2f3b sites

Given the diversity of roles we have observed for E2f3 in regulating neural precursor and neurogenesis-related functions (Mcclellan et al., 2007; 2009; Julian et al., 2013), we wondered if individual E2f3 isoforms bind unique target genes. We developed an experimental approach that allowed us to identify genomic regions bound by E2f3a alone, E2f3b alone, and by both E2f3a and E2f3b with the use of a single ChIP antibody. We have previously confirmed that neurospheres deficient in one E2f3 isoform do not up-regulate the remaining isoform, and that the C-terminal specific antibody used in these experiments (Fig. 3.1C) precipitates both E2f3 isoforms in our neurosphere cultures with comparable efficiencies (Fig. 3.1B and (Julian et al., 2013)).

Binding sites unique to E2f3b were defined as those peaks that were enriched in both the WT and E2f3a-/- ChIP-chip experiments, but were absent in the E2f3b-/- experiment. Similarly, E2f3a-specific sites were those peaks present in both the WT and E2f3b-/- experiments, but absent in E2f3a-/- cells. Finally, E2f3a/b common peaks were present in all three experiments. By ensuring that all targets included in our isoform-dependent data sets are also present in WT cells, we have excluded any targets that may be aberrantly bound in

the absence of one E2f3 isoform by the remaining isoform (we identified 537 and 93 such peaks in E2f3a-/- and E2f3b-/- cells, respectively (Fig. 3.1E)). This helped to ensure that only high confidence binding events, reflective of the normal situation in WT cells, are considered.

We identified 3144 enriched E2f3 peaks (corresponding to 2979 unique target genes) in E2f3a-deficient cells (Fig, 3.1E), suggesting that E2f3b is present at the majority of E2f3-bound sites in NPCs. Alternatively, we found only 1595 E2f3 peaks (1518 unique target genes) in E2f3b-/- cells, demonstrating that E2f3a is present at a much smaller subset of E2f3-bound sites. Our comparative analysis revealed 2665 high confidence E2f3 enriched peaks (Fig. 3.1F), where peaks are enriched in more than one experimental condition. Half of these sites are common to both E2f3 isoforms (49.3%), while another 46.1% are unique to E2f3b. With only 4.6% of E2f3 sites bound specifically by E2f3a, it is clear that this isoform shows little specificity in its binding interactions and is generally only enriched at sites that are also bound by E2f3b. In our comparisons, we also identified 779 peaks that were enriched for E2f3 in WT cells only (Fig. 3.1E). We believe that these peaks are not false positives, but instead represent a class of genes that require the presence of both E2f3 isoforms (i.e. a maximum amount of E2f3 protein) for optimal ChIP detection. This is supported by the fact that 78% of these E2f3 'unique' sites overlap with peaks in E2f3a-/- and E2f3b-/- cells for which an E2f3 binding signal was obtained but did not meet our FDR requirement (Supplemental Fig. 3.2), and that these targets comprise a significant proportion of E2f3 target genes that are most highly expressed in a cluster of neuronal tissues (see below, and Supplemental Fig. 3.3A).

Our finding that E2f3a is enriched at only a subset of E2f3b bound sites in NPCs

substantiates similar findings in myoblasts (MBs) (Asp et al., 2009), where a lack of E2f3a enrichment at a significant number of E2f3b bound sites was postulated to arise from decreased epitope affinity of an E2f3a specific antibody used in that study. Our data demonstrate that E2f3a truly is enriched at only half of E2f3b-bound sites and binds very few promoters that aren't also bound by E2f3b. This highlights E2f3a as a unique member of the E2f family, as such a limited repertoire of target genes has not been observed for other E2f family members (Xu et al., 2007). E2f4 binding sites, however, overlap significantly with both E2f3a (85% of E2f3a peaks overlap with E2f4 peaks) and E2f3b (67% of E2f3b peaks overlap with E2f4 peaks) binding sites (Fig. 3.1G), demonstrating that most E2f bound promoter sites in NPCs are bound by at least two different E2f proteins.

E2fs bind a network of target genes involved in diverse neural precursor cell fate decisions

To identify the biological processes with which E2f target genes are associated in NPCs, and to determine if E2f3a, E2f3b and E2f4 potentially control unique functions, we performed gene ontology (GO) analysis of the identified target genes (Ashburner et al., 2000). We found that E2f target genes are highly enriched in previously characterized E2f-dependent functions, including the DNA damage response, cell cycle regulation, chromatin organization, microtubule-based processes, gene expression, and cell death (Fig. 3.2 and Table 3.1). These biological processes were similarly enriched between both common and specific E2f3 and E2f4 target genes, aside from a few categories for which E2f3 specific targets show reduced or no enrichment. Examples of previously established E2f targets that we identified, most common to both E2f3 and E2f4, include *Wee1*, *Pcna*, *Ccne*, *Bard1*,

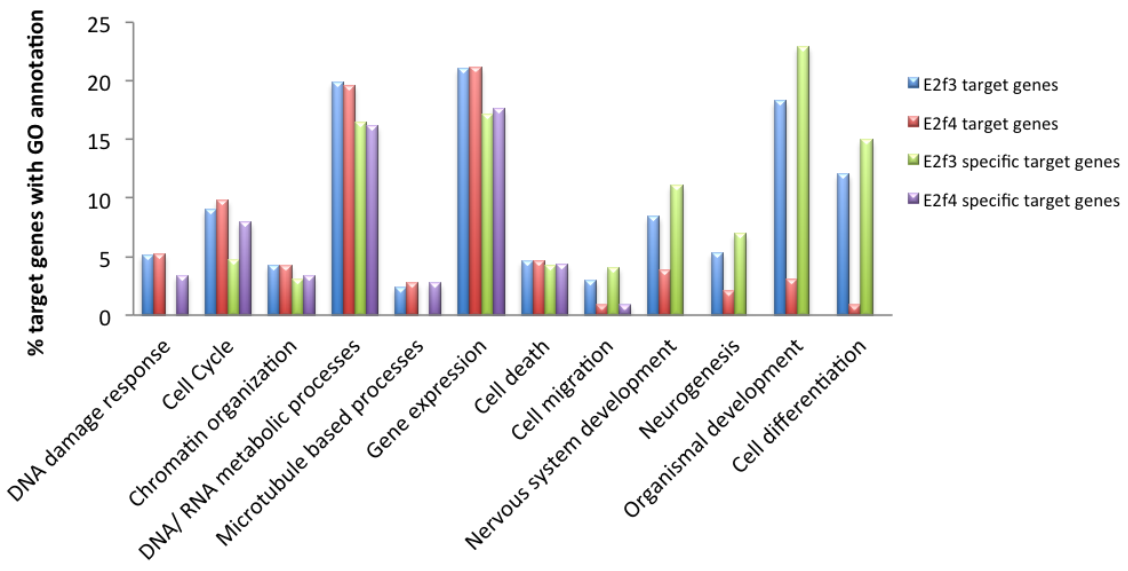


Figure 3.2. E2f3 and E2f4 target genes control common and unique cellular functions
Gene Ontology (GO) analysis of E2f3 and E2f4 target genes, expressed as the percentage of all target genes in each group (groups indicated to the right of the graph) with a particular GO annotation (GO annotations indicated at the bottom of the graph). Target genes were identified as the gene whose TSS is closest to each E2f peak.

GO term	Gene count (E2f3; E2f4)	% of all genes (E2f3; E2f4)	*p*-value (E2f3)	Fold enrichment (E2f3)
DNA damage response	178; 201	5.17%; 5.27%	8.68E-55	3.53
Cell cycle	312; 376	9.06%; 9.86%	6.51E-79	3.05
Chromatin organization	148; 162	4.3%; 4.25%	6.43E-29	2.73
DNA/ RNA metabolic processes	685; 809	19.89%; 19.66%	9.10E-118	2.38
Microtubule based processes	81; 105	2.35%; 2.75%	1.82E-10	2.37
Gene expression	724; 809	21.11%; 21.21%	7.13E-123	2.35
Cell death	159; 177	4.62%; 4.64%	2.89E-13	1.93
Cell migration	104; 11	3.02%; 0.9%	6.63E-07	1.87
Nervous system development	291; 148	8.45%. 3.88%	1.37E-17	1.71
Neurogenesis	184; 79	5.34%; 2.07%	5.78E-08	1.62
Organismal development	631; 119	18.32%; 3.12%	8.97E-31	1.56
Cell differentiation	405; 34	12.08%; 0.89%	6.16E-17	1.54

Table 3.1. Quantification of select gene ontology processes among genes targeted by E2f3 and E2f4

Table displaying the number and percent of genes bound by either E2f3 or E2f4 belonging to particular GO annotations, as indicated by GREAT. The *p*-values are generated using the hypergeometric test with correction for multiple hypothesis testing using the Benjamini-Hochberg algorithm. The following *p*-values are applied: (*p<1.0E-4, **p<1.0E-10, ***p<1.0E-20). Fold enrichment is calculated based on the number of E2f3 bound genes with a given annotation compared to the number of genes in the background set with that annotation. The file used to generate genes for 'background' comparisons in these calculations was all known mouse promoter regions that were surveyed on our DNA microarrays.

Rad51, *Brca1/2*, *Ezh2* and members of the pRb-E2f pathway (*Rb1*, *Rbl1*, *E2f1*, *E2f3*) (Table 3.2).

We also observed a strong enrichment in functions related to differentiation and development, including nervous system dependent processes (Fig. 3.2 and Table 3.1). Remarkably, E2f3 bound target genes are much more highly enriched than E2f4 targets for these processes (specifically: organismal development, cell differentiation, cell migration, nervous system development and neurogenesis), while most 'classical' E2f-dependent functions are equally represented between E2f3 and E2f4 targets. Quantification of the number of genes bound in specific categories demonstrates that E2f3 targets 12-fold more differentiation-related genes (405 versus 34 genes) and 2-fold more nervous system development and neurogenesis related genes (291 versus 148 genes; 184 versus 79 genes) than does E2f4 (Table 3.1). Interestingly, we found that migration-related genes are almost exclusively targeted by E2f3, but not E2f4. Genes in the migration category predominantly include those coding for proteins that participate in actin dynamics and structural reorganization of the cytoskeleton, extracellular guidance molecules, cell surface receptors and signaling proteins (including members of the Netrin, Ephrin and Reelin pathways), and cellular adhesion proteins (Table 3.2).

Analysis of the target genes themselves (see Table 3.2 for examples) suggests that E2fs may regulate a number of distinct neural precursor cell fates and related processes, including proliferation, self-renewal, maintenance of precursor populations and differentiation. These functions include regulation of classical cell cycle pathways, growth factor pathways, chromatin modifications, apoptosis, and transcription factors that regulate stem cell identity as well as neurogenesis. We were particularly intrigued by the observation

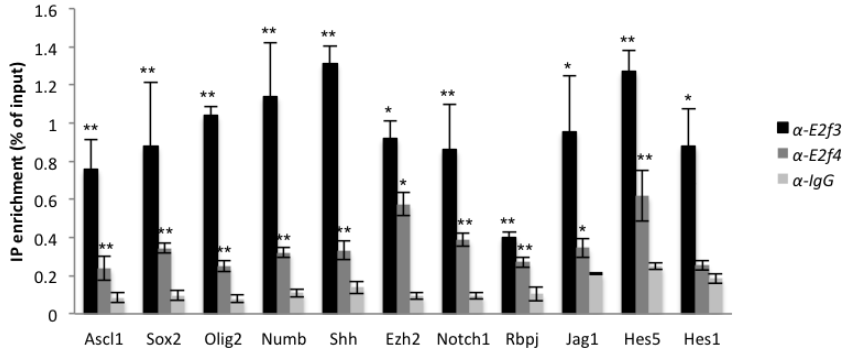
GO Term	Target gene examples
Cell Cycle	**Common genes:** *Wee1, Cdkn1a, PCNA, Gas1/2, Cdc2/25a, E2f1, Rb1, Rbl1, Ccna1/2, Ccne1/2, Anapc5/13, Topbp1, Mis12, Gmnn* **Unique to E2f3:** *Fmn2, Ing4, Lats2* **Unique to E2f4:** *Rbl2, Cdkn3, E2f2, E2f8*
Gene expression	**Common genes:** *Yy1, HDAC3, E2f3, Polr2a, Crtc1, Cebpa, Cited2, Prim1, Myst2, Smarcb1, Eif3b, E4f1, Nfya, Sp1, Tcf3, Ctcf* **Unique to E2f3:** *Klf3, Mxi1, Eif5, Onecut3, Pcgf2* **Unique to E2f4:** *Eif6, Cebpa, Tbp, Atf3, Ash2l*
DNA damage response	**Common genes:** *Gadd45a, Exo1, H2afx, Brca1, Mlh1, Msh2, Mutyh, Bard1* **Unique to E2f3:** *Chk1, Rad50, Rad51, Nhej1* **Unique to E2f4:** *Rad21, Brca2, Xrcc3*
Chromatin organization	**Common genes:** *Rbl1, Rbbp4, Dnmt1, Smarca2, Eed, Ezh2, Epc1, Mll1, Suv39h1/2, Ctcf, Mbd1* **Unique to E2f3:** *Mecp2, Cbx2, Epc2, Atrx, Gadd45b* **Unique to E2f4:** *Suz12, Rbbp7*
Cell death	**Common genes:** *Casp3, Fadd, E2f1, Bcl10, Xiap* **Unique to E2f3:** *Bax, Trp53, Bcl2, Casp8* **Unique to E2f4:** *Casp9, Dffa, Rad21, Aifm1*
Cell differentiation	**Common genes:** *Sema5b, Id1, Rb1, Epc1, Smarcb1, Ascl1, Fzd5, Dll4, Gsk3b, Wnt6, Apc, Tcf3, Tgfbr1, Bmp7, Smad4/7, Acvr2b* **Unique to E2f3***: Fgfr1, Ctnna2, Ntng1, Vegf, Id2-4, Axin1/2, Bmp4, Smad3* **Unique to E2f4:** *Smad6*
Cell migration	**Common genes:** *Myh9, Cenpe, Tuba1b, Apoe* **Unique to E2f3:** *Efnb1/2, Cdh2, Nrp1, Nup62, Ctnna2, Cd47, Dab1, Elmo2, Mapt, Pcnt, Rtn4, Lamc1, Lamb2, Ntn1, Unc5c, Rgma, Vegf, Fgfr1, Sema3g* **Unique to E2f4:** *Hap1, Nde1, Tuba1c, Lama1*
Nervous system development/ neurogenesis	**Common genes:** *Sox2, Notch1, Hes5, Jag1, Rbpj, Numb, Numbl, Ezh2, Shh, Gli3, Ptch1, Olig2, Ascl1, Pax6, Lhx2, Gdnf, Fgfr2/3* **Unique to E2f3:** *Hes1, Hhip, Egfr, Fgfr1, Fmr1, Mecp2, Vegf, Ntn1, Sema6c, Lhx1, Wnt5a* **Unique to E2f4:** *Notch3, Gli2, Hes2, Wnt7a/b*

Table 3.2.

Select gene ontology classifications enriched among E2f target genes in NPCs, with examples of target genes both common and unique to E2f3 and E2f4.

that, although E2f3 binds a substantially higher number of neurogenesis, development and differentiation related gene promoters than does E2f4, many fundamental regulators of NPC fate decisions are shared by E2f3 and E2f4. These common targets include genes or members of pathways that have previously been described as targets of the pRb-E2f family in the brain, such as *Sox2* (Li et al., 2012; Julian et al., 2013), members of the Notch/Hes pathway (*Notch1, Hes5, Jag1, Rbpj, Numb*) (Vanderluit et al., 2004; 2007b), the Fgf pathway (*Fgfr2* and *Fgfr3*) (Mcclellan et al., 2009), and the Sonic Hedgehog (Shh) pathway (*Shh, Gli3, Ptch1, Rbpj,* and *Jag1*) (Ruzhynsky et al., 2007). Other key E2f3/E2f4 targets include members of the Wnt (*Wnt6, Fzd5, Dll4, Gsk3b, Apc,* and *Tcf3*) and Transforming growth factor beta (Tgf-b) signaling pathways (*Tgfbr1, Bmp7, Smad4&7,* and *Acvr2b*), the transcription factors *Ascl1, Pax6* and *Lhx2,* and the chromatin regulators *Dnmt1, Mbd1* and members of the Polycomb/ Trithorax families (*Ezh2, Eed, Epc1,* and *Mll1*). E2f3 specific targets include additional growth factors and extended family members of the core pathways that are represented by E2f3/E2f4 common targets, as well as the important chromatin regulators *Mecp2* and *Atrx*. We confirmed binding of E2f3 and E2f4 to the promoters of a group target genes that play pivotal roles in NPC fate decisions, using quantitative ChIP (Fig. 3.3A). We note that the majority of these genes show enrichment for both E2f4 and at least one E2f3 isoform, generally with tightly overlapping peaks, and that these regions largely correspond with evolutionarily conserved regions (Fig. 3.3B). Thus, E2fs bind a wide range of target genes related to NPC cell fate decisions, and most of these common targets are bound by more than one E2f protein.

A


IP enrichment (% of input)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ascl1
Sox2
Olig2
Numb
Shh
Ezh2
Notch1
Rbpj
Jag1
Hes5
Hes1
α-E2f3
α-E2f4
α-IgG

B

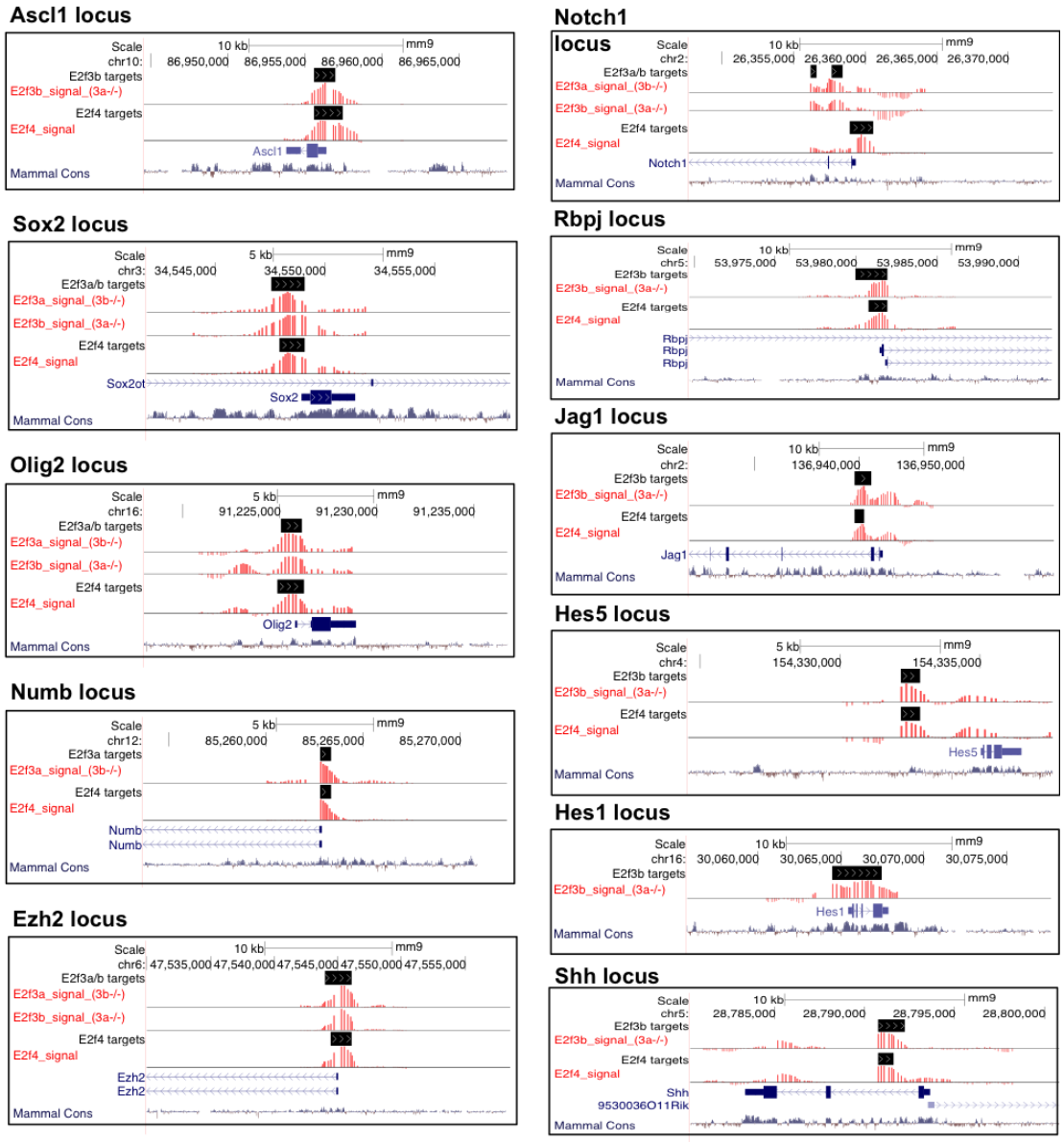

Ascl1 locus
Sox2 locus
Olig2 locus
Numb locus
Ezh2 locus
Notch1 locus
Rbpj locus
Jag1 locus
Hes5 locus
Hes1 locus
Shh locus
Scale
mm9
E2f3b targets
E2f3b_signal_(3a-/-)
E2f4 targets
E2f4_signal
Mammal Cons
Ascl1
Sox2ot
Sox2
Olig2
Numb
Ezh2
E2f3a targets
E2f3a_signal_(3b-/-)
E2f3a/b targets
Notch1
Rbpj
Jag1
Hes5
Hes1
Shh
9530036O11Rik

Figure 3.3. E2f3 and E2f4 bind an overlapping set of gene promoters that control fundamental neural precursor cell fate decisions

(A) qChIP analysis validates E2f3 and E2f4 enrichment, compared to IgG, at binding sites identified by ChIP-chip corresponding to a panel of genes involved in neural precursor cell fate decisions. Both E2f3 and E2f4 are enriched at at all analyzed sites, except for that corresponding to the gene *Hes1*. (*$p<0.05$, **$p<0.01$). The average of at least three biological replicates are shown +/- SEM.

(B) Binding-peak profiles for E2f3a (E2f3 ChIP-chip in E2f3b-/- neurospheres), E2f3b (E2f3 ChIP-chip in E2f3a-/- neurospheres) and E2f4 (ChIP-chip in wild-type cells), generated through the UCSC Genome Browser (http://genome.ucsc.edu/). Profiles are shown for all E2f family members that had a significantly enriched peak(s) at the indicated loci (E2f identity indicated to the left). Relative levels of mammalian conservation for genomic sequences are shown along the bottom of each image ('Mammal Cons'). The majority of E2f3 peaks are located within highly conserved regions, and most promoters are bound by multiple E2fs with highly overlapping peaks. Peaks are generally found overlapping or directly proximal to the TSS, except for *Notch1*, *Hes1* and *Shh* peaks (peaks downstream from the TSS), and *Hes5* (peaks substantially upstream from the TSS).

E2f3a and E2f3b target genes control common cellular processes in NPCs

An analysis of E2f3 isoform dependent functions and target genes in myoblasts revealed specificity for E2f3b in attenuating the expression of genes involved in muscle differentiation and for E2f3a in regulating cell cycle and proliferation related genes (Asp et al., 2009). Contrary to these findings, we have previously observed that E2f3a promotes and E2f3b inhibits neuronal differentiation, demonstrating that both E2f3 isoforms impact differentiation in NPCs and suggesting that they may target different classes of promoters in this cell type compared to those in MBs. Interestingly, GO analysis demonstrated that the percentage of E2f3a-specific, E2f3b-specific and E2f3a/b common target genes in NPCs are similarly enriched for the majority of E2f3-dependent functional gene categories (Supplemental Fig. 3.4 and Supplemental Table 3.1), including the categories 'cell cycle' and 'cell differentiation' that were differentially represented by E2f3 isoforms in MBs. Considering the total number of genes bound in each category, a model emerges whereby the majority of E2f3 sites are enriched for E2f3b and roughly half of these are also enriched for E2f3a, but the two isoforms are equivalent in terms of the functional categories of the genes that they target in NPCs.

In the case of the 'neurogenesis' and 'nervous system development' E2f3 targets we discovered, 70% of this group of genes is targeted either exclusively by E2f3b (39.2%) or by both E2f3a and E2f3b (31.2%), while only very few are targeted specifically by E2f3a (3.5%) or by E2f3 in WT cells only (25.3%) (Fig. 3.4). Notably, among the genes identified as specific to E2f3b are members of the Fgf signaling pathway, which we have previously shown is regulated by E2f3 (Mcclellan et al., 2009), suggesting that E2f3b may uniquely regulate Fgf signaling. On the other hand, as was the case for E2f3 and E2f4 common

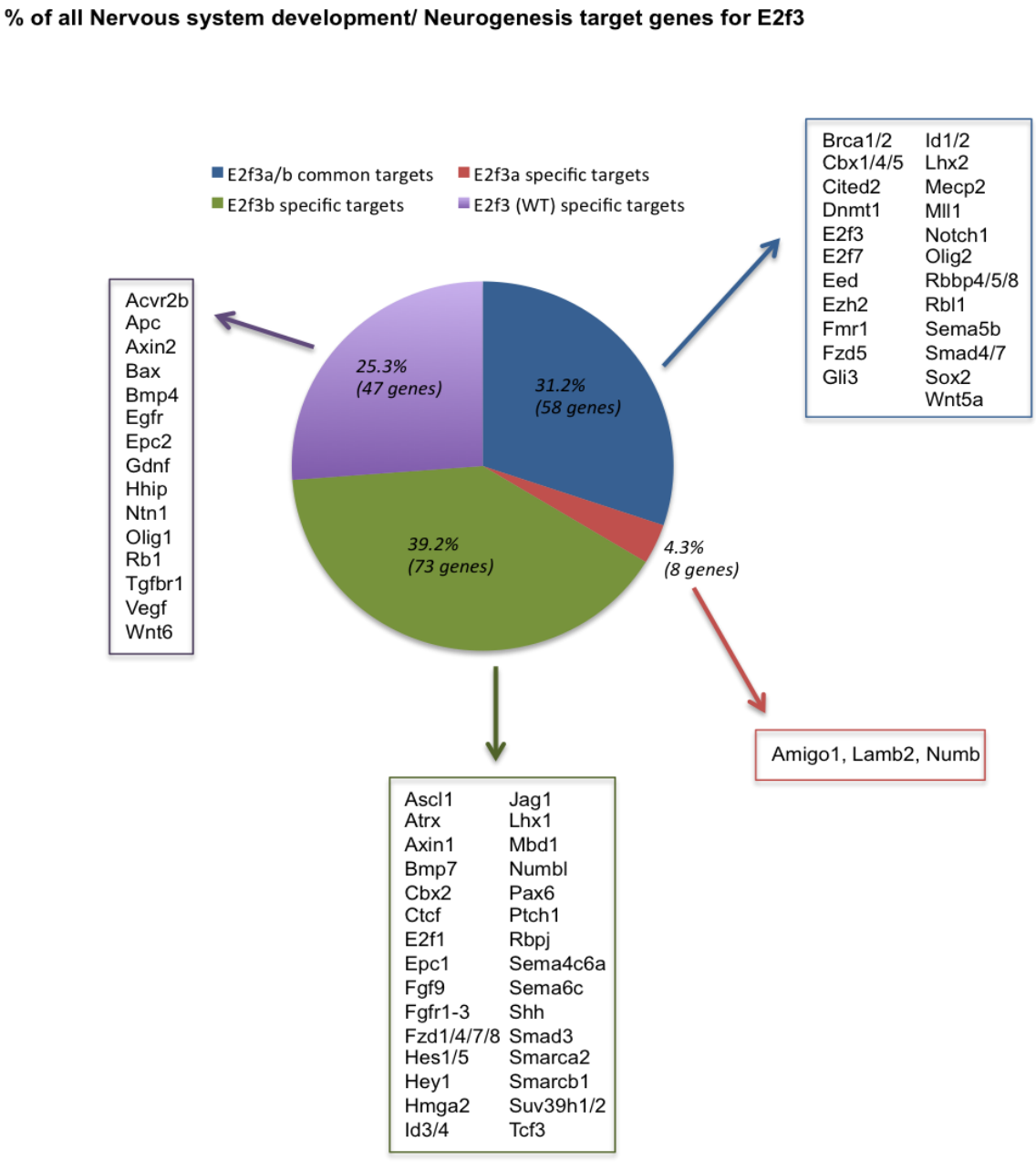


Figure 3.4. Genes involved in nervous system development and neurogenesis are targeted by both E2f3 isoforms

The number of E2f3 target genes with the GO classifications of 'nervous system development' or 'neurogenesis' was determined, and the percentage of these genes that belong to specific groups of E2f3a and E2f3b targets are shown. This demonstrates that the distribution of nervous system related target genes is similar to the distribution for all E2f3 targets: the majority are bound by E2f3b without about half of these sites also bound by E2f3a; another 25% are bound by E2f3 in wild-type cells only. Examples of genes belonging to each category of E2f3 targets are listed.

targets, we found that many fundamental regulators of NPC fate decisions are shared between E2f3a and E2f3b. This group includes core members of the Polycomb repressive complex 2 (*Ezh2* and *Eed*), chromatin regulators *Brca1*, *Brca2*, *Dnmt1*, *Mll1* and *Mecp2*, and key regulators of precursor self-renewal and differentiation such as *Sox2*, *Notch1 Olig2*, *Lhx2* and *E2f3* itself (see Figure 3.4 for more examples of E2f3 isoform specific and common target genes). These findings, along with those for E2f4, further highlight that the majority of E2f-bound targets in NPCs may be regulated by multiple E2f proteins.

E2f proteins overlap at the TSS of transcriptionally active genes in NPCs

The E2f binding peaks we identified are strongly localized to TSS regions: while we surveyed the region from -5kb to +3kb relative to the TSS, 88-90% of enriched peaks located within 600bp upstream and 600bp downstream of a TSS (Fig. 3.5A). Based on their expression patterns throughout the cell cycle, their ability to interact with pocket proteins, and their activity in transcriptional reporter assays, E2f3a has classically been considered to be a transcriptional activator, while E2f3b and E2f4 are thought to be repressors. However, recent evidence has demonstrated that these classifications are not always upheld in a physiological setting and that individual E2f proteins can function as both activators and repressors (Tsai et al., 2008; Asp et al., 2009; Chong et al., 2009b; Lee et al., 2011). Indeed, we have recently demonstrated that E2f3a counter-intuitively represses, while E2f3b activates, expression from the *Sox2* locus in NPCs (Julian et al., 2013), which is indicative of at least some degree of antagonistic transcriptional activity by E2f3 isoforms in NPCs. We therefore sought to determine if E2f3 isoforms exhibit clear differences in their

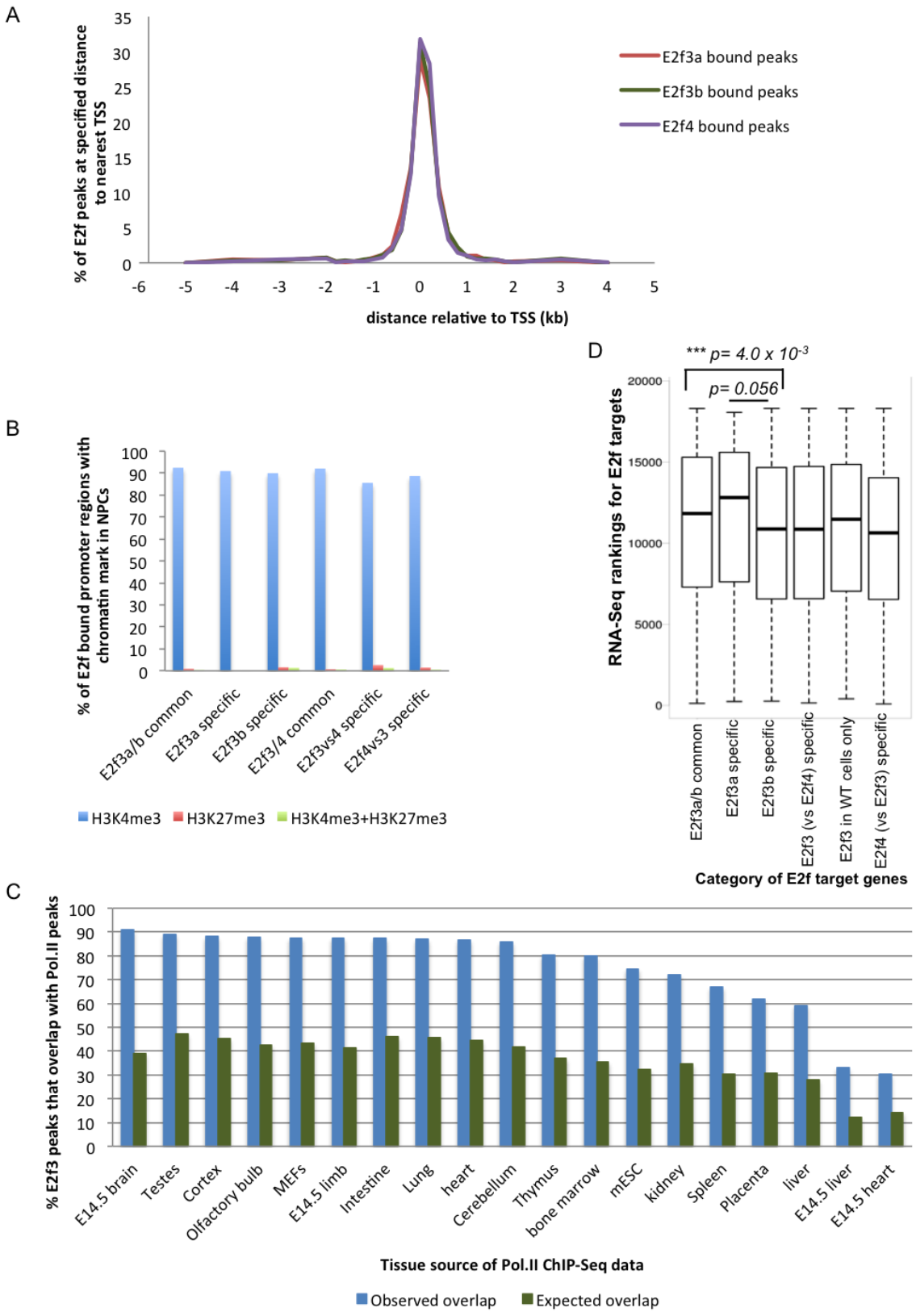

A
% of E2f peaks at specified distance to nearest TSS
distance relative to TSS (kb)
E2f3a bound peaks
E2f3b bound peaks
E2f4 bound peaks
B
% of E2f bound promoter regions with chromatin mark in NPCs
E2f3a/b common
E2f3a specific
E2f3b specific
E2f3/4 common
E2f3vs4 specific
E2f4vs3 specific
H3K4me3
H3K27me3
H3K4me3+H3K27me3
D
*** p= 4.0 x 10-3
p= 0.056
RNA-Seq rankings for E2f targets
E2f3a/b common
E2f3a specific
E2f3b specific
E2f3 (vs E2f4) specific
E2f3 in WT cells only
E2f4 (vs E2f3) specific
Category of E2f target genes
C
% E2f3 peaks that overlap with Pol.II peaks
E14.5 brain
Testes
Cortex
Olfactory bulb
MEFs
E14.5 limb
Intestine
Lung
heart
Cerebellum
Thymus
bone marrow
mESC
kidney
Spleen
Placenta
liver
E14.5 liver
E14.5 heart
Tissue source of Pol.II ChIP-Seq data
Observed overlap
Expected overlap

Figure 3.5. E2fs are similarly enriched at the TSS of transcriptionally active genes in neural precursors

(A) Distribution of E2f3a, E2f3b and E2f4 bound sites relative to the nearest TSS. Data was categorized into 200bp bins, and the percent of bound sites in each category (indicated in upper right corner) within each bin range are plotted. E2fs bind highly overlapping regions in NPCs.

(B) Promoter regions were generated by obtaining sequences lying 1000bp on either side of the TSS of all known mouse genes. The percentage of promoter regions bound by E2f3 in our experiments that also contained the chromatin marks H3K4Me3 or H3K27Me3 (or both) in NPCs (Mikkelsen et al., 2007) was calculated. Overlaps were identified using UCSC Genome Browser. The vast majority of E2f bound sites, regardless of the category of E2f bound genes (indicated along the X-axis), also posses the activating H3K4Me3 modification.

(C) The percentage of E2f3 bound promoter regions that also contain Pol.II peaks in the indicated tissues (Shen et al., 2012) (along the X-axis) was quantified and plotted. The expected overlap between E2f3 and Pol.II at promoter regions was calculated as the percentage of all known mouse promoters surveyed in our experiments at which Pol. II is bound.

(D) RNA-Seq reads were obtained for individual transcripts expressed in E14.5 brain tissue (Shen et al., 2012), and transcripts were ranked in ascending order of read density. Boxplots display ranked expression data for genes belonging to distinct classes of E2f3 and E2f4 targets (gene classes indicated along the X-axis), and show interquartile ranges (IQR) with whiskers representing data points up to 1.5 times the IQR boundaries. p-values were determined in R by Wilcoxon rank sum test with continuity correction.

transcriptional properties. To investigate this, we compared our E2f peaks with ChIP-chip data obtained in NPCs for chromatin marks associated with transcriptional activation and repression (Mikkelsen et al., 2007)and with ChIP-Seq data obtained for RNA Polymerase II (Pol.II) binding in E14.5 brain and other tissues (Shen et al., 2012). We anticipated that the differential binding patterns of E2f3a and E2f3b may correspond to distinct transcriptional activities and chromatin environments, however we observed that the majority of promoter regions containing any combination of E2f proteins carry marks of transcriptionally active chromatin. 85-92% of E2f bound promoter sites posses the H3K4Me3 chromatin modification (Fig. 3.5B) (the expectation across all promoters for the presence of H3K4Me3 in NPCs is just 51.3%) and over 90% of E2f3 peaks overlap with Pol.II peaks (Fig. 3.5C) (general expectation for Pol. II at promoters is 39-45% across neuronal tissues) (results are similar for all combinations of E2f bound targets (data not shown)), indicative of transcriptional activation or regions poised for activation. In contrast, on average only 1.3% of E2f bound promoters possess the repressive H3K27Me3 modification and 0.7% are bivalent (Fig. 3.5B), which is lower than the expected overlaps of 3.2% (H3K27Me3) and 1.3% (bivalent), based on the prevalence of these chromatin modifications at all known promoters in NPCs.

In fact, we find that the presence of an E2f protein is a common feature of a large fraction of active promoters in NPCs, similar to previous findings in cell lines for E2f1, E2f4 and E2f6 (Xu et al., 2007), as 43% and 47% of promoter regions containing H3K4Me3 (in NPCs) and Pol.II (in E14.5 brain) are enriched for at least one E2f3 or E2f4 protein. Alternatively, only 12% of promoters containing the H3K27Me3 modification are bound by an E2f factor. Given that 26% of all known promoters are bound by E2f3 or E2f4, these

figures suggest that E2fs exhibit a strong preference for binding to active promoters, and are excluded from silenced regions.

In a further effort to determine if individual E2fs are associated with unique transcriptional properties, we determined the relative expression levels of genes whose promoters are bound by different combinations of E2f3a, E2f3b and E2f4 proteins in E14.5 brain tissue (Shen et al., 2012). We found that levels of gene expression were similar for targets common to both E2f3a and E2f3b, specific to E2f3 (versus E2f4) specific, specific to E2f4 (versus E2f3) specific, and bound by E2f3 in WT cells only (Fig. 3.5D). However, our analysis of E2f3a/b common and isoform specific targets demonstrated a pattern suggesting that the presence of E2f3b is associated with reduced levels of gene expression compared to E2f3a. The group of E2f3b specific target genes showed significantly reduced expression compared to E2f3a/b common targets, and although not reaching statistical significance, E2f3a specific target genes appear to be expressed on average at higher levels than E2f3b specific genes (Fig. 3.5D). This is consistent with a more potent activator role for E2f3a and repressor function for E2f3b, demonstrating that although both E2f3 isoforms are similarly bound to active promoter regions, they can influence transcription in different ways. Analysis of E2f3 target genes that are highly expressed or that show reduced expression in a cluster of neuronal tissues (Fig. 3.7) does not, however, support the notion that E2f3a functions strictly as a transcriptional activator and E2f3b solely as a repressor. Both the high and low expression gene classes exhibit similar proportions of genes whose promoters are bound by E2f3b alone, by both E2f3a and E2f3b, and by E2f3 in WT cells only (Supplemental Fig. 3.3A). These distributions are similar as for all E2f3 bound targets and suggest that, although the average expression levels of their entire set of target genes reflect

transcriptional differences, both E2f3 isoforms can contribute to gene activation and repression. These data support the notion that, on a broad scale, E2f3a more potently activates while E2f3b represses transcription, relative to one another, but that neither isoform can be strictly defined as an activator or repressor and that both can contribute to these functions.

Extensive tissue-specific promoter binding by E2f3 isoforms in NPCs

Analysis of Pol.II occupancy in a panel of tissues (Shen et al., 2012) demonstrates that a large percentage of our E2f3 bound sites are also bound by Pol.II not only in embryonic brain (91% of our E2f3 sites posses Pol. II in this tissue), but also in adult brain (88% posses Pol. II in adult cortex and olfactory bulb) and additional tissue types (eg. testes (89%), lung (87%), thymus (80%)) (Fig. 3.5C). The percentage of E2f3 sites bound by Pol.II is substantially lower, however, in a number of other tissue types; for example, only 67%, 33% and 30% of our E2f3 sites also posses Pol.II in spleen, embryonic liver and embryonic heart tissue, respectively (Fig. 3.5C). This suggests that E2f3 bound sites are differentially regulated depending on the cell type and/or that E2fs can exhibit significant tissue specificity in their genomic binding patterns, contrary to previous reports which demonstrated little tissue specificity of binding sites for E2f1 and E2f4 (Conboy et al., 2007; Xu et al., 2007).

To directly determine if E2f3 isoforms bind to their target genes in a tissue specific manner, we compared the E2f3 bound peaks we discovered in NPCs with those identified in MBs (Asp et al., 2009). In this analysis, we compared only those E2f3 peaks that were discovered using the same α-E2f3 antibody (sc-878) and that fell within genomic regions that were surveyed in both the NPC and MB experiments. We found a surprisingly low

degree of overlap, with just 30% of MB genes also identifying as targets in neural precursors (NP) (Fig. 3.6A). Strikingly, GO analysis revealed a strong conservation of classical E2f processes between the two cell types (specifically: cell cycle, gene expression, DNA damage response, chromatin organization, and cell death). In contrast, tissue-specific E2f3 targets were enriched for developmentally related functions, and these categories are poorly represented within the group of genes targeted by E2f3 in both NPs and MBs (Fig. 3.6B). These developmental functions are highlighted by a strong NP enrichment for 'cell differentiation', 'nervous system development' and 'neurogenesis' related processes, while MB specific targets are primarily enriched in functions related to 'cell adhesion', ' response to wounding' and 'skeletal system development'. These findings demonstrate both a conservation of 'classical' E2F-dependent function between cell types, which occurs through a relatively small proportion of E2f3 binding sites, as well as extensive cell type specificity in terms of both target gene binding and function.

We identified a cluster of over 400 E2f3 target genes that are highly expressed and a large cluster of over 850 E2f3 targets with reduced expression in brain, compared to other adult tissues (Fig. 3.7). We also identified a strong cluster of genes highly expressed specifically in testis. Additionally, a few other cell types (mainly ESCs, osteoblasts, macrophages and a panel of immune system cells) showed moderately increased expression across a similar panel of genes. These expression profiles generally correlate with high levels of Pol.II enrichment at E2f3 bound sites in these cell types (Fig. 3.5C). Interestingly, the genes showing up-regulation across multiple cell types largely correspond to the cluster of genes with low expression in neuronal tissues, while highly expressed genes in the brain are

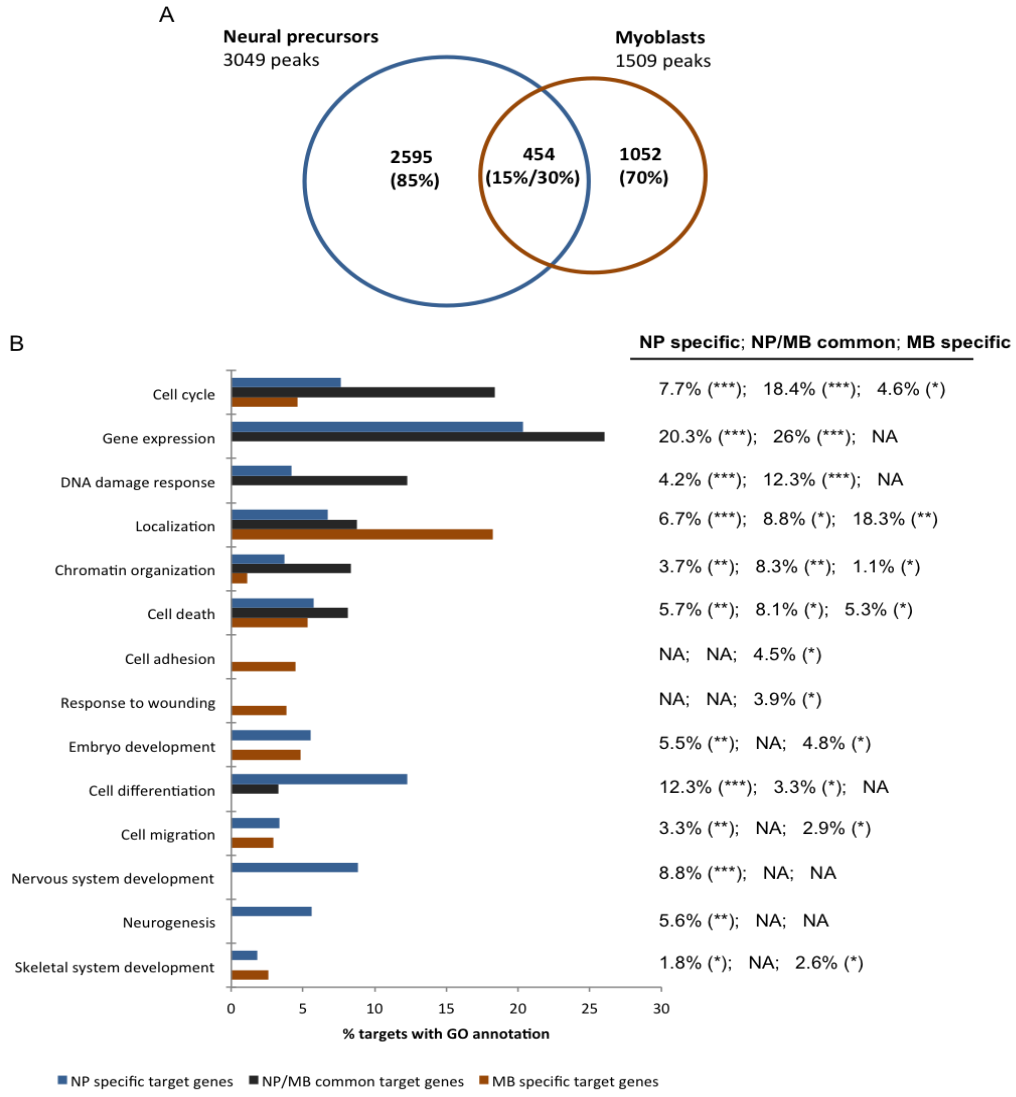


Figure 3.6. E2f3 binds predominantly unique target genes in neural versus muscle precursors

(A) The number of peaks identified for E2f3, using a common pan-E2f3 antibody, in neural precursors (NPCs) and myoblasts (MBs) that overlap with regions surveyed in both NPC and MB experiments were calculated. The overlap between NPC and MB E2f3 peaks was calculated using UCSC Genome Browser. Numbers of common and unique peaks are shown, with percentages of NPC and MB peaks in each group in parentheses. 85% of E2f3 peaks in NPCs and 70% of MB peaks are specific to that tissue type.

(B) GO analysis of NPC and MB common and specific genes. The percentage of genes from each category with specific functional annotations (indicated along the Y-axis) is graphed, and indicated in the table to the right of the graph. p-values are also indicated (*p<1.0E-4, **p<1.0E-10, ***p<1.0E-20). E2f3 specific peaks in NPCs are uniquely enriched in processes related to differentiation and development.

predominantly specific to this tissue (Fig. 3.7). These observations further substantiate our finding of extensive tissue specificity for E2f3 target genes.

E2f3 and CTCF are co-enriched at the promoters of neural specific target genes

We analyzed the E2f3 bound regions discovered by our ChIP-chip experiments for the occurrence of binding sites of other known transcription factors, using the Cisgenome program. This approach has been shown to help uncover combinatorial regulation partners of transcription factors. As expected, we found a very strong over-representation of sequence motifs corresponding to E2F factors (Fig. 3.8A). Interestingly, we also found a high occurrence of NF-Y and Sp1 binding sequence elements, as well as a specific enrichment for binding motifs for the CTCF transcription factor (Fig. 3.8A). The presence of a large number of Sp1 and NF-Y sites is consistent with the fact that these transcription factors are known to regulate cell cycle related gene expression (Elkon, 2003). On the other hand, the over-representation of CTCF binding sequences was more unexpected. Using a dataset of genomic CTCF sites identified in E14.5 brain tissue by ChIP-Seq (Shen et al., 2012), we uncovered a remarkable overlap between binding sites of the two factors: 41.9% of our E2f3-bound peaks directly overlap with CTCF-bound regions. This is not simply due to a tendency for both factors to reside near the TSS of genes: while only 19.4% of known genes have an E2f binding site within 3kb upstream and 1kb downstream of their TSS and 34.9% have a CTCF binding site, we found that 51.4% of E2f3-bound promoter regions also have a CTCF binding site, which is significantly greater than the expected overlap (Fig. 3.8B).

Interestingly, we observed a similar level of enrichment for CTCF at E2f3 bound regions in adult olfactory bulb as we did in E14.5 brain tissue, but the enrichment level was

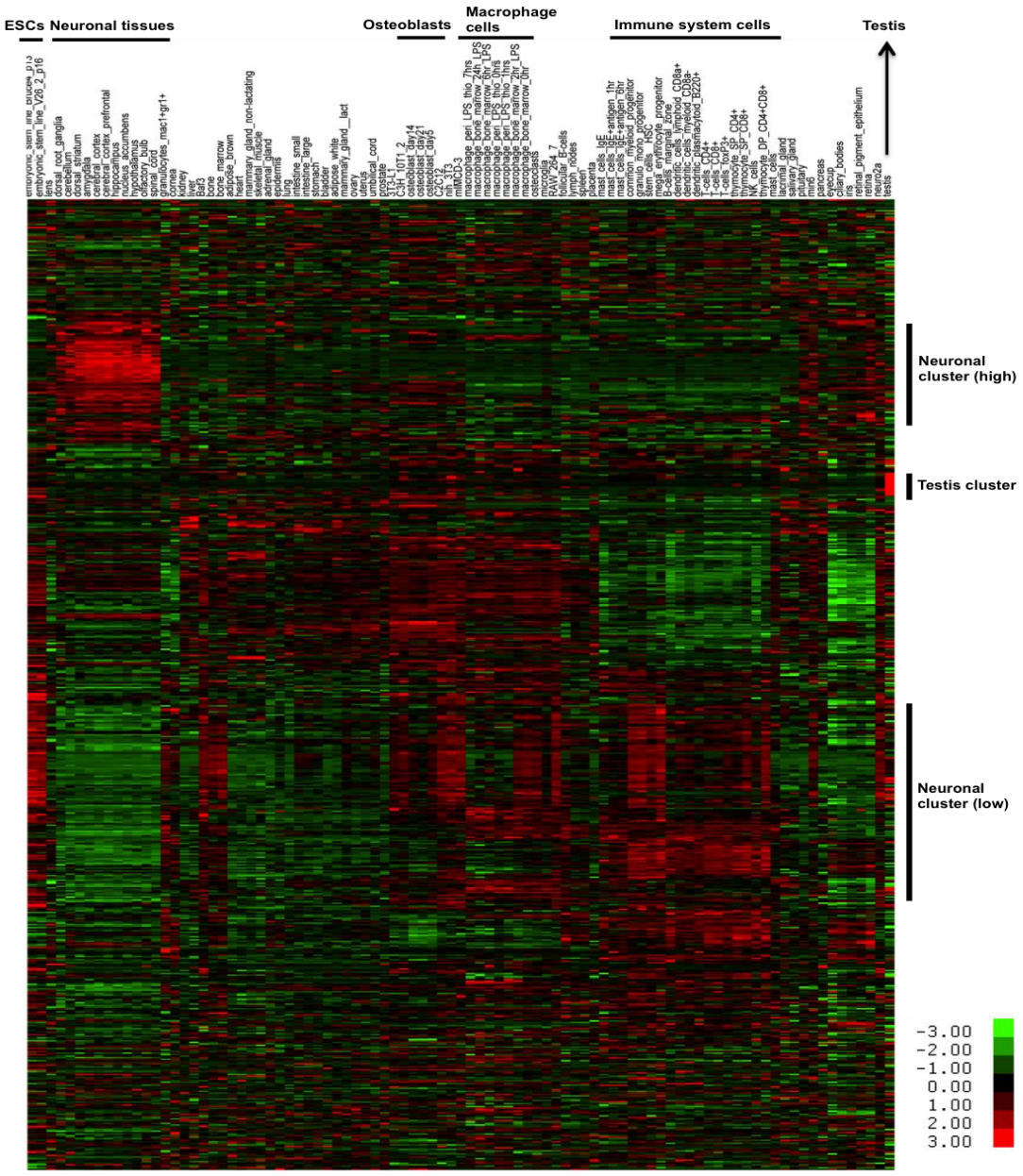


Figure 3.7. Expression profile of E2f3 target genes across multiple tissues
Expression profiles were extracted for all E2f3 target genes discovered in NPCs that are represented in the mouse Gene Atlas GNF3 study. Data were normalized and then processed to an average of 0 and a standard deviation of 1. Data were then clustered using k-means, with the Euclidean metric, which grouped the data into gene and tissue/cell type clusters. Predominant clusters are indicated along the right hand side (genes found in neuronal and testis tissues), and additional tissues/ cell types with moderate to high patterns of gene expression are indicated along the top of the image. The legend indicates the colour profile related to normalized gene expression values. ESCs = embryonic stem cells.

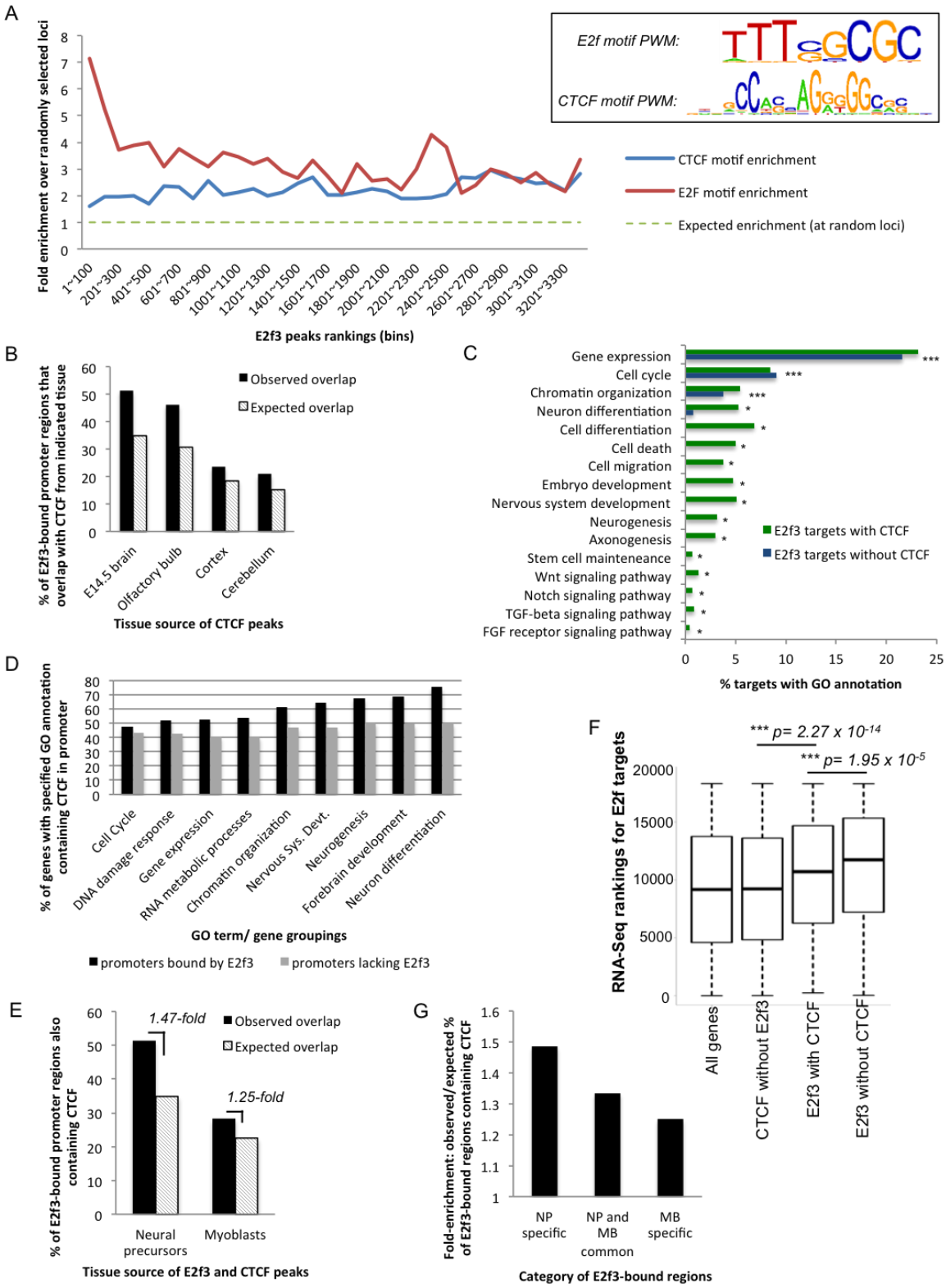

A
E2f motif PWM:
CTCF motif PWM:
Fold enrichment over randomly selected loci
CTCF motif enrichment
E2F motif enrichment
Expected enrichment (at random loci)
E2f3 peaks rankings (bins)
B
% of E2f3-bound promoter regions that overlap with CTCF from indicated tissue
Observed overlap
Expected overlap
E14.5 brain
Olfactory bulb
Cortex
Cerebellum
Tissue source of CTCF peaks
C
Gene expression
Cell cycle
Chromatin organization
Neuron differentiation
Cell differentiation
Cell death
Cell migration
Embryo development
Nervous system development
Neurogenesis
Axonogenesis
Stem cell mainteneance
Wnt signaling pathway
Notch signaling pathway
TGF-beta signaling pathway
FGF receptor signaling pathway
E2f3 targets with CTCF
E2f3 targets without CTCF
% targets with GO annotation
D
% of genes with specified GO annotation containing CTCF in promoter
Cell Cycle
DNA damage response
Gene expression
RNA metabolic processes
Chromatin organization
Nervous Sys. Devt.
Neurogenesis
Forebrain development
Neuron differentiation
GO term/ gene groupings
promoters bound by E2f3
promoters lacking E2f3
F
*** p= 2.27 x 10-14
*** p= 1.95 x 10-5
RNA-Seq rankings for E2f targets
All genes
CTCF without E2f3
E2f3 with CTCF
E2f3 without CTCF
E
1.47-fold
1.25-fold
Observed overlap
Expected overlap
% of E2f3-bound promoter regions also containing CTCF
Neural precursors
Myoblasts
Tissue source of E2f3 and CTCF peaks
G
Fold-enrichment: observed/expected % of E2f3-bound regions containing CTCF
NP specific
NP and MB common
MB specific
Category of E2f3-bound regions

Figure 3.8. E2f3 and CTCF are co-enriched at nervous system related genes
(A) Enrichment of E2f and CTCF position weight matrices (PWM) (PWMs indicated at top right) within E2f3 peaks discovered in NPCs. The genomic loci enriched for E2f3 were ranked in decreasing order of ChIP-chip enrichment and were binned into groups of 100 regions. The number of E2f and CTCF PWMs within each binned group, and within a control set of randomly selected loci, was quantified and results are represented as the fold enrichment of E2f or CTCF PWMs in E2f3 bound regions over random loci.
(B) Promoter and surrounding regions containing 3kb upstream to 1kb downstream of TSSs were extracted for all known mouse promoters from the RefSeq database. The percentage of promoter regions at which E2f3 is bound in NPCs that also contained CTCF in in different brain tissues (indicated along the X-axis) was quantified, and expressed as 'observed overlap'. The 'expected overlap' is the percentage of all known promoter regions surveyed in our ChIP-chip experiments that contain a CTCF peak.
(C) Comparative GO analysis of E2f3 bound target genes that contain CTCF versus those without CTCF. The percentage of E2f3 targets in each group with specific GO annotations (indicated on y-axis) was determined by GREAT. Target genes enriched for both E2f3 and CTCF are uniquely enriched for differentiation, development, and cell fate related processes. *p*-values are indicated (*p<1.0E-4, **p<1.0E-10, ***p<1.0E-20).
(D) The percent of target genes whose promoter region is bound by CTCF that belong to different GO annotations (indicated along x-axis) were calculated separately for targets either with or without E2f3. CTCF bound genes involved in nervous system-dependent processes, particularly 'neuron differentiation', show the greatest co-enrichment for E2f3.
(E) The percentage of promoter regions containing E2f3 in either neural precursors or myoblasts that also contain CTCF was calculated as the 'observed overlap'. The 'expected overlap' is the percentage of all known promoter regions surveyed in either the NPC or MB ChIP-chip experiments that contain a CTCF peak. Fold-enrichment of observed over expected overlaps is indicated. E2f3 bound sites are more highly enriched for CTCF binding in NPCs versus MBs.
(F) Box plots of ranked RNA-Seq data in E14.5 brain for gene promoters at which only CTCF is present ('CTCF without E2f3'), E2f3 is present ('E2f3 without CTCF'), or both are present in ('E2f3 with CTCF') NPCs. Ranked expression data for all genes surveyed in our ChIP-chip experiments ('All genes') is also shown for comparative purposes. p-values were determined in R using the Wilcoxon rank sum test with continuity correction.
(G) The percentage of promoter regions at which E2f3 is bound specifically in neural precursor cells ('NP specific'), specifically in myoblasts ('MB specific') or in both NPs and MBs ('NP and MB common') at which CTCF is also bound was calculated ('observed' enrichment). Data is presented as the fold increase of the observed over expected enrichment of CTCF at E2f3-bound promoters. The expected enrichment was calculated as the percentage of all promoter regions surveyed in ChIP-chip experiments at which CTCF is bound.

substantially lower in other adult brain tissues (cortex and cerebellum) (Fig. 3.8B). As E14.5 brain and the olfactory bulb are regions that undergo extensive neurogenesis, in contrast to the other tissues surveyed, this finding raised the possibility that CTCF enrichment may be most predominant at E2f3 bound sites that contribute to the regulation of neural precursor cell fate related processes during neurogenesis. Supporting this hypothesis, GO analysis demonstrated that while E2f3 target genes are equally represented for 'cell cycle', 'chromatin organization' and 'gene expression' categories whether or not CTCF is also bound, targets at which both E2f3 and CTCF are bound are uniquely enriched for functions that impact cell fate related processes, many with important roles during neurogenesis (differentiation, migration, cell death, neuron differentiation, axonogenesis, stem cell maintenance) and specific pathways that impact the maintenance and expansion of neural precursor populations (Wnt, Notch, TGF-beta and FGF signaling pathways) (Fig. 3.8C). Furthermore, promoter regions with E2f3 have a greater likelihood of also containing CTCF than those without E2f3 if they are involved in functions related to brain development or neuron generation, while genes that regulate cell cycle or gene expression related processes are bound by CTCF to a similar degree regardless of E2f3 status (Fig. 3.8D). For example, 'cell cycle' genes contain CTCF at 47% and 43% of promoter regions with and without E2f3 respectively, while 'neuron differentiation' genes demonstrate a remarkable 76% overlap with CTCF at E2f3 bound sites and only 50% overlap at promoter regions lacking E2f3.

E2f3 and CTCF bound regions identified in myoblasts demonstrated a much lower degree of overlap compared to neural precursor identified binding sites, as well as a very low level of enrichment over the expected degree of overlap based on random genomic binding (Fig. 3.8E). Furthermore, genes whose promoters are bound by both E2f3 and CTCF in

NPCs are expressed on average at significantly higher levels than all known genes or those bound only by CTCF, and E2f3-bound promoters that do not contain CTCF have even higher expression levels (Fig. 3.8F). This demonstrates that co-enrichment of E2f3 and CTCF has a significant affect on gene expression, and that the transcriptional potency of both factors is influenced by this association. Together, these data suggest that a strong overlap between E2f3 and CTCF may be a feature specific to neural tissue and may at least partially account for E2f3-dependent target gene binding and/or gene regulation in NPCs. Further supporting this possibility, E2f3-bound regions that were identified only in neural precursors but not in myoblasts are more highly enriched for CTCF binding compared to E2f3-bound regions common to both NPs and MBs, and even more-so for those unique to MBs only (Fig. 3.8G). These data demonstrate an unexpected association between E2f3 and CTCF, most highly enriched at the promoters of genes that regulate cell fate decisions in neural precursor cells, and implicate CTCF as a potential regulatory co-factor of E2f3 in the transcriptional control of tissue-specific target genes in NPCs.

DISCUSSION

Through our genome-wide identification of binding sites for E2f3a, E2f3b and E2f4 in promoter regions, we show evidence of both E2f specific and common target genes in neural precursor cells, both among the E2f proteins themselves in NPCs and in a tissue specific manner. We have unveiled a class of previously unappreciated E2f target genes with regulatory roles in diverse NPC fate choices, specifically self-renewal, maintenance of precursor identity, precursor proliferation, cell death, and neuron generation. Our data demonstrate that although E2f3 targets are more highly enriched in differentiation and nervous system related process compared to E2f4 targets, many fundamental regulators of NPC functions are shared between them and most target genes are bound by multiple E2f factors (combinations of E2f3a, E2f3b and E2f4). We further show that E2f3 isoforms contribute to similar cellular functions in NPCs, contrary to their roles in myoblasts, but that they exhibit extensive tissue specificity in the target genes that they bind. Finally, we demonstrate a surprising overlap between E2f3 bound sites and the CTCF transcription factor, predominantly at genes involved in regulating nervous system functions, suggesting a potential tissue-specific regulatory co-factor for E2f3 in NPCs.

We found that E2fs target the promoters of over 3000 genes in neural precursor cells, hundreds of which play a role in processes related to nervous system development. A number of phenotypes have been previously observed for the pRb-E2f pathway *in vivo* using knockout mouse models in the context of NPC regulation and neurogenesis, and a small group of E2f-dependent target genes that contribute to these phenotypes have been characterized (Vanderluit et al., 2004; Ruzhynsky et al., 2007; Vanderluit et al., 2007a;

Mcclellan et al., 2009; Andrusiak et al., 2011; Ghanem et al., 2012; Julian et al., 2013). We show here, however, that this class of genes is targeted by E2fs much more extensively than previously thought, suggesting that the E2f family is a wide-spread regulator of diverse neural precursor cell fate processes. Confirming previous studies, we identified as E2f-dependent target genes through our screen: *Sox2* (Julian et al., 2013) and members of the Fgf (Mcclellan et al., 2009), Notch (Vanderluit et al., 2004; 2007b) and Shh pathways (Ruzhynsky et al., 2007). When we originally identified the Shh pathway as a target of E2f4 and the Notch pathway as a p107/E2f target, we were unable to determine the specific gene(s) within these pathways that contributed to pocket protein/ E2f knockout phenotypes. Our data now reveals that, in fact, multiple members of these pathways are E2f targets (Shh pathway: *Shh, Gli1-3, Ptch1*; Notch pathway: *Notch1*, *Hes1*, *Hes5*, *Jag1, Rbpj*) and therefore they may be disrupted in more than one way when E2f levels are altered. Furthermore, although the majority of functional categories are equally represented among target genes of different E2f family members, multiple members of the Fgf signaling pathway are uniquely bound by E2f3b, suggesting specificity of function for E2f3b in this particular context.

Based on our previous findings that demonstrated a repressive role for E2f3a and an activator role for E2f3b at the *Sox2* promoter and opposing phenotypes in knockout mouse models (Julian et al., 2013), we hypothesized that E2f3a and E2f3b may broadly function in NPCs uncharacteristically as a repressor and an activator, respectively. Our expression analysis of E2f-bound target genes demonstrated that, on average, E2f3 isoforms do exhibit opposing transcriptional activities; however, this data reflected the classical roles of E2f3 as an activator and E2f3b as a repressor. Furthermore, the group of genes bound by both E2f3 isoforms were expressed at a level in between that of E2f3a and E2f3b specific targets,

suggesting that there are a wide-range of target genes in addition to *Sox2* that may be regulated by E2f3 isoforms in an opposing manner to potentially moderate levels of gene expression. We were unable to determine how many of these genes may be repressed by E2f3a and activated by E2f3b as at the *Sox2* promoter, however future studies focused on evaluating expression levels of these genes following modification of E2f3 isoform levels should help to clarify this issue. However, we also found that E2f3a and E2f3b are present in comparable proportions at the promoters of genes that are either highly expressed or repressed in a cluster of neuronal tissues, and that therefore both isoforms are associated with transcriptional activation and repression. These data suggest that E2f3a and E2f3b may function more as modifiers of gene expression in NPCs as opposed to strict activators and repressors. This type of 'modifier' regulation has been suggested previously for E2f3b in the transcriptional attenuation of target genes during myogenic differentiation (Asp et al., 2009), and we now show that both E2f3 isoforms appear to function in this manner in actively proliferating NPCs.

Our data suggests that E2F3 isoforms are more extensive regulators of differentiation and development-related functions than are other E2F family members. This is evidenced by the finding that E2f3 binds to the promoters of 12, 5, 2 and 2-fold more genes compared to E2f4 with the GO annotations of 'cell differentiation', 'organismal development', 'nervous system development' and 'neurogenesis', respectively. Previous studies of E2F genomic binding patterns have not reported large differences in the functional annotations and identity of bound target genes among multiple E2F family members, aside from the analysis of E2f3 targets in myoblasts, which revealed that E2f3b has a preferential role compared to E2f3a in targeting differentiation-related genes. These observations, combined with our findings,

suggest a unique role for E2f3 isoforms in cellular differentiation-related processes. Additionally, the finding that E2f3 almost exclusively targets migration related genes compared to E2f4 substantiates our previous findings of a role for E2f3 in pRb-mediated neuronal migration and further suggests that E2f3 may specifically regulate this function (Mcclellan et al., 2007). Accordingly, the increased enrichment of E2f3 over E2f4 at differentiation related genes could explain why NPCs deficient in E2f3 but not E2f4 exhibit neuronal differentiation defects (Ruzhynsky et al., 2007; Julian et al., 2013).

This increased representation of differentiation and development related genes among E2f3 targets also appears to underlie a tissue-specific genomic binding pattern that has not been previously observed among the E2F family. Expression analysis of our E2f3 target genes identified a cluster of genes that are highly expressed specifically in neural tissues, as well as a second group of genes whose repression is also largely unique to these tissues. This demonstrated that a sub-set of E2f3 target genes we identified in NPCs may be uniquely regulated in the brain, countering previous reports of largely redundant target genes for E2Fs among different tissues and cell types (Xu et al., 2007). We were also surprised to find that 85% and 70% of E2f3-bound targets in NPCs and myoblasts respectively were unique to that tissue type. This is the first study to demonstrate such a substantial degree of specificity among genomic binding sites for any E2F factor in different tissue types, suggesting that E2F3 is uniquely capable of considerable flexibility in the genomic regions that it targets. This phenomenon does not appear to be the result of differential isoform usage by E2f3 in NPCs versus MBs, but to true differences in the genomic regions targeted, as the common α-E2f3a/b antibody used in both experiments pulled down binding sites of both E2f3a and E2f3b, with E2f3b-bound sites making up the majority in both cell types (our data and (Asp

et al., 2009)). Interestingly, our analysis of the functional categories of E2f3 target genes in NPCs and MBs suggests that E2f3 isoforms may exhibit differential transcriptional activities between the two cell types, as E2f3b was shown to be necessary for the promotion of differentiation in MBs while E2f3a plays no apparent role, and E2f3b inhibits neuronal differentiation in NPCs while E2f3a promotes this activity (Asp et al., 2009; Julian et al., 2013). As our study in NPCs and the aforementioned study in MBs are the only reports of genome-wide binding site analysis of E2f3 isoforms currently in the literature, it will be important for future studies to examine E2f3 binding sites in other tissues. These studies are necessary to determine if this phenomenon of tissue-specific gene targeting by E2f3 is unique to the NPC/ MB comparison or if it is indeed more wide-spread, and if transcriptional regulation by E2F3 is an important facet of tissue-specific differentiation programs in general.

We identified an unexpected association between E2f3-bound promoter regions and presence of the CCCTC binding factor (CTCF). Interestingly, this association appears to be preferentially enriched in neural versus muscle precursors, and even moreso at the promoters of E2f3-bound genes involved in neurogenesis and nervous system developmental functions. This pattern suggests that CTCF could participate with E2F3 in binding to tissue-specific genomic sites in neural precursor cells. CTCF is most well understood as an insulator protein in mammals, functioning predominantly in intergenic and intronic regions to block the activity of enhancers (Phillips-Cremins and Corces, 2013). ChIP-Seq studies have recently demonstrated, however, that 20-25% of CTCF bound sites are located within proximal promoter regions (Shen et al., 2012), and the localization of CTCF to these regions has been linked to the regulation of alternative promoter selection as well as the pausing of RNA

Polymerase II at transcriptional start sites and sites linked to mRNA spliceosome assembly (Shukla et al., 2011; Paredes et al., 2012; Phillips-Cremins and Corces, 2013). Furthermore, a pair of recent studies has identified E2F binding motifs as over-represented among CTCF-bound regions in a number of mammalian cell types, although the functional consequences of this association were not investigated (Martin et al., 2011; Whitfield et al., 2012). This suggests the possibility that the E2f3-CTCF connection we have uncovered may be relevant in other cell types. Although our results demonstrate a potential regulatory co-factor for E2F3 in the regulation of neurogenesis-related genes, future studies are required to determine if this phenomenon is unique to NPCs or if it is more wide-spread, and to determine which cellular role CTCF plays at these sites.

In conclusion, our studies implicate the E2F family as important regulators of a diversity of neural precursor cell fate decisions by regulating an extensive number of target genes with direct roles in these processes. We have discovered previously unidentified target genes for the E2F family that suggest novel functions, and have uncovered a remarkable tissue-specificity for E2f3 isoforms, shedding light on the mechanisms by which cell cycle regulatory proteins impact NPC fate decisions.

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MATERIALS AND METHODS

Mouse Models and Cell Culture

Germline E2f3a and E2f3b deficient mice were generated originally by G. Leone and were maintained on an FVB/N background (Chen et al., 2007; Tsai et al., 2008). Animal dissections were approved by the University of Ottawa's Animal Care Committee, which abides by the Canadian Council on Animal Care guidelines.

All cells used in this study were cultured as neurospheres. Neural precursors were obtained by dissection of the ganglionic eminence from developing embryos at gestational age E14.5, and cells were cultured as neurospheres as previously described (Vanderluit et al., 2004). E2f3a-/- and E2f3b-/- NPCs were obtained from crosses of either two heterozygous or two homozygous (knock-out) animals.

Classical ChIP

Chromatin immunoprecipitation assays were performed and quantified as previously described (Julian et al., 2013), using 2ug of specific antibody and 20ug of chromatin for each reaction. ChIP data from wild-type cells are from a combination of E2f3a+/+ and E2f3b+/+ experiments; we observed no obvious discrepancies in levels of chromatin enrichment with any primers pair assessed in wild-type cells from either colony. All chromatin was prepared from animals between the ages of 2 and 4 months old. Statistical analysis of ChIP data was performed using unpaired two-tailed t tests, with differences considered significant with a *p*-value of <0.05 (*), $p<0.01$ (**), and $p<0.001$ (***).

ChIP-chip Design, Experimental Set-up and Data Analysis

ChIP-chip experiments were performed as previously described (Liu et al., 2010). Briefly, pilot experiments were performed using DNA microarrays on which all non-repetitive sequences from mouse chromosome 7 were represented. Based on the results of these experiments, we designed microarrays, manufactured by Agilent Technologies, which contained DNA probes representing 5kb upstream to 3kb downstream of the TSS of all known mouse transcripts, based on the mm9 mouse genome assembly. A total of 24654 unique regions were surveyed. The 60-mer probes were typically tiled at a density of 5 per kilobase of DNA sequence. UCSC gene annotations, extracted from the UCSC genome browser (http://genome.ucsc.edu/), were used to identify all known transcripts. ChIP-chip experiments were each performed in triplicate. Following ChIP, IP and whole cell extract (WCE) DNA fragments were PCR amplified and labeled with Cy3 and Cy5 dyes, as previously described (Acosta-Alvear et al., 2007). The WCE DNA serves as a reference control for determining levels of DNA enrichment in the ChIP experiments. 12.5ug of labeled DNA were hybridized overnight to DNA arrays along with 30ug of mouse C0t-1 DNA. Slides were washed and then scanned at 2um resolution on an Agilent laser scanner, and data extraction was performed using Agilent Feature extraction 10.1. Median fluorescence values were obtained and grouped for replicate experiments and data were normalized (via the quantiles method) and analyzed using the CisGenome program (Ji et al., 2008). Using CisGenome, IP enrichments were determined using a moving average (MA) calculation with a half-window size of 3 probes located within a maximum of 750 base pairs of either side of the central probe. Enriched peaks were identified as regions of at least 100bp that contain at least 5 probes that received an MA score of at least 2 (where the enrichment

score is at least 2-fold over that for the WCE control signal in that region). Enriched peaks located within a 1000bp region were merged into a single peak, as they were likely to result from a single binding event.

Analysis of Enriched DNA Motifs

To determine the enrichment of E2F and CTCF consensus binding motifs in our E2f3-bound peaks, we obtained a position weight matrix (PWM) corresponding to a known consensus motif for each of these factors. The E2F and CTCF PWMs were obtained from the Transfac database. E2f3-bound peaks were separated into bins of 100, ranging from highest to lowest enrichment values, and the number of E2F and CTCF motifs present in each binned category was determined. The number of motifs present among randomly selected loci was also determined, and the enrichment of E2F and CTCF PWMs in our E2f3-bound peaks over the random loci was calculated.

Expression Clustering Analysis

We determined the expression profile of E2f3 target genes discovered in our ChIP-chip analysis across a panel of over 90 mouse tissues and cell lines. Gene expression data for all E2f3 target genes in NPCs was obtained from the mouse Gene Atlas V3 (Lattin et al., 2008), and was analyzed and normalized as previously described (Liu et al., 2010). k-means clustering was performed on the gene expression data using the Cluster 3 program, and was visualized using Java Treeview, as previously described (Liu et al., 2010), to identify tissues and cell types that demonstrate similar gene expression profiles.

Gene Ontology, Assignment of E2f Target Genes, and Peak Analysis

Gene ontology (GO) classifications were determined through GREAT (http://bejerano.stanford.edu/great/public/html/index.php) and the DAVID Bioinformatics Database (http://david.abcc.ncifcrf.gov/), and levels of enrichment were determined through GREAT by comparing groups of E2f target genes with a control set of genes. Target genes were defined as the gene with the closest transcriptional start site to each E2f binding peak (at a maximum distance of 10kb) or to background control loci, which were all regions surveyed in the ChIP-chip experiments. Significance values were obtained using the hypergeometric test with correction for multiple hypothesis testing via the Benjamini-Hochberg algorithm. Differences were considered significant with a p-value of <0.05. Lists of E2f3 target genes belonging to specific GO categories were extracted from GREAT.

The number of overlapping or unique peaks between different factors was determined by comparing genomic coordinates of enriched peaks through the UCSC Table Browser. Promoter regions bound by a particular factor were determined through Table Browser by first extracting promoter regions that were surveyed in ChIP-chip experiments (specified as 3000bp upstream and 1000bp downstream of the TSS of all known transcripts for E2f3 comparisons with CTCF, and 1000bp upstream and downstream of TSS sites for all other comparisons), and then determining the overlap between promoter regions and the genomic coordinates of binding peaks for the factor in question.

Additional Sources of ChIP-chip and ChIP-Seq Data

Data for the H3K4Me3 and H3K27Me3 chromatin modifications in neural precursor cells was obtained from (Mikkelsen et al., 2007), who used ChIP-Seq to determine the genomic

enrichment of these chromatin marks in neural precursor cells induced from mouse embryonic stem cells. Genome-wide enrichment of Pol. II and CTCF in both E14.5 brain and other indicated tissue types was obtained from (Shen et al., 2012). E2f3 binding peaks at promoter regions in myoblasts was obtained from ChIP-chip experiments from (Asp et al., 2009). Finally, data for CTCF genomic binding sites is freely available as part of the ENCODE consortium, and is published here (Landt et al., 2012).

E2f Target Gene Expression Profiling

We obtained RNA-Seq read data (FPKM values) for all transcripts expressed in whole brain tissue at E14.5 (Shen et al., 2012), and ranked transcripts in ascending order of read density. E2f or CTCF target genes were determined using GREAT as the genes whose TSS is closest to each enriched binding peak (with a maximum distance of 10kb), and the target genes from each category were assigned FPKM values and expression rankings. Boxplots displaying the ranked expression data were generated in R, and p-values were determined by Wilcoxon rank sum test with continuity correction. These procedures are modified from (Wamstad et al., 2012).

Antibodies and Primers

Identical E2f and IgG antibodies were used for ChIP-chip and classical ChIP experiments in this study. These include rabbit specific antibodies against E2f3, E2f4 and normal rabbit IgG (Santa Cruz) as a control antibody for ChIP.

The following primer pairs were used to amplify enriched chromatin at the promoters of the indicated genes (‘For’= forward primer; ‘Rev’ = reverse primer).

Ascl1 For: 5’-GACTCCCGGCTGAATAAACA-3’; Rev: 5’-TCCCTTTGCCACTTTTTCTG-3’

Chrna1 For:5’-CACAATGAAAAGACCATCCAGA-3’;Rev:5’-ATGTACTTCACGCCCTCGAT-3’

Ezh2 For: 5’-GCAAATAGCTCACCCCATGT-3’; Rev: 5’-GGGATTTTGCCATTCAGATG-3’

Hes1 For: 5’-CAGCTCCCAAGTTGTTACTGC-3’; Rev: 5’-ACCGGTGCTAAACCACTGAC-3’

Hes5 For: 5’-CTGCCCCCTCAACTACTGTC-3’; Rev: 5’-CTCACACCATCCGACTTGAG-3’

Jag1 For: 5’-GGTGGGAACTGGAAGTAGCA-3’; Rev: 5’-CGAGCCCTATATTGCCTTGA-3’

Notch1 For: 5’-GGATGAGCCATTCAGCAGTT-3’; Rev: 5’-CCAGGTTGCTCTCAAGGACT-3’

Numb For: 5’-CCCCATACCACACTTCCAAC-3’; Rev: 5’-GAACTGCCCAATCATCAACC-3’

Olig2 For: 5’-CTGCAGCAACTGCCACTAAG-3’; Rev: 5’-AGAATGAACACCGAGGTTGC-3’

p107For:5’-TTAGAGTCCGAGGTCCATCTTCT-3’;Rev:5’-GGGCTCGTCCTCGAACATATCC-3’

Rbpj For: 5’-CGGTTTTCCTCAGTCTCCAC-3’; Rev: 5’-GATCTGCTCACTGCCTTTCC-3’

Shh For: 5’-CCTCCTCACACCTTTCCAAG-3’; Rev: 5’-CCGACCACCTTAAATCTGGA-3’

Sox2 For: 5’-CCCATTTATTCCCTGACAGC-3’; Rev: 5’-CTCTTCTTTCTCCCAGCCCTA-3’

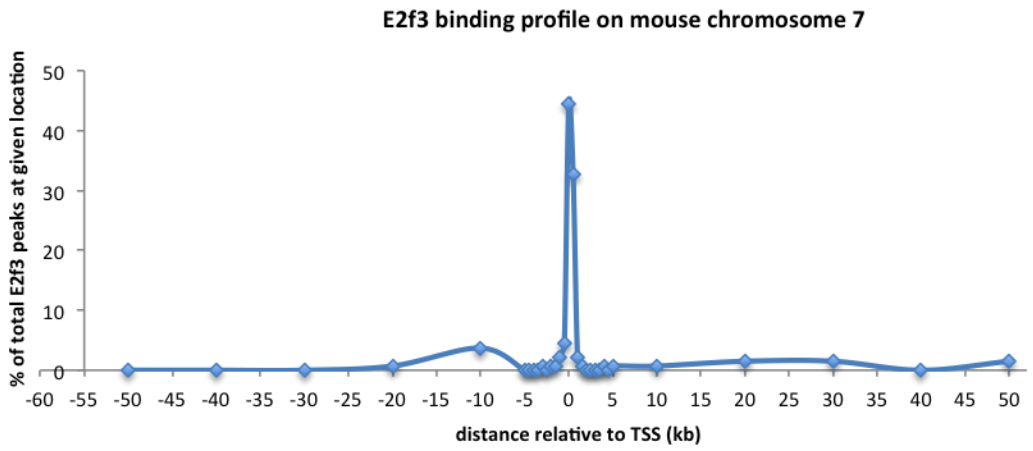
TK1 For: 5’-GAAGAAATCTGGCGCTCAAC-3’; Rev: 5’-AGCTACGTGAAAAGCCTGGA-3’

SUPPLEMENTAL DATA

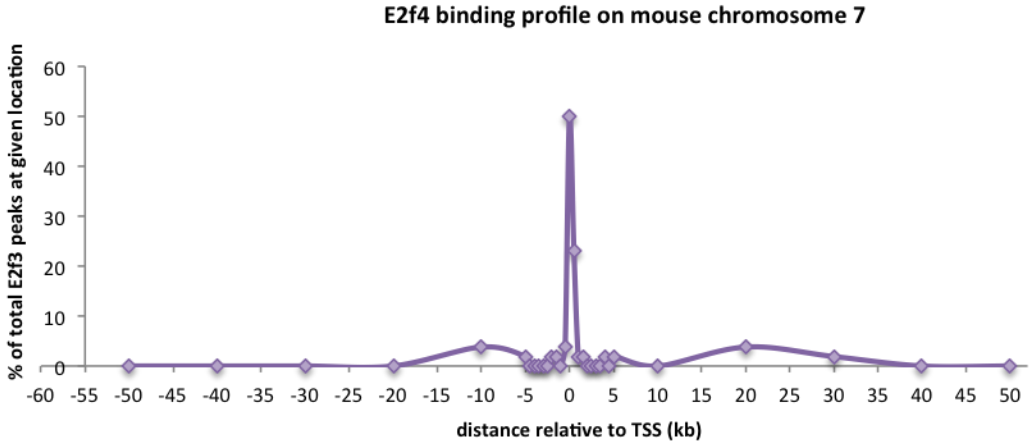
Supplemental Figures 3.1 – 3.4

Supplemental Table 3.1

A

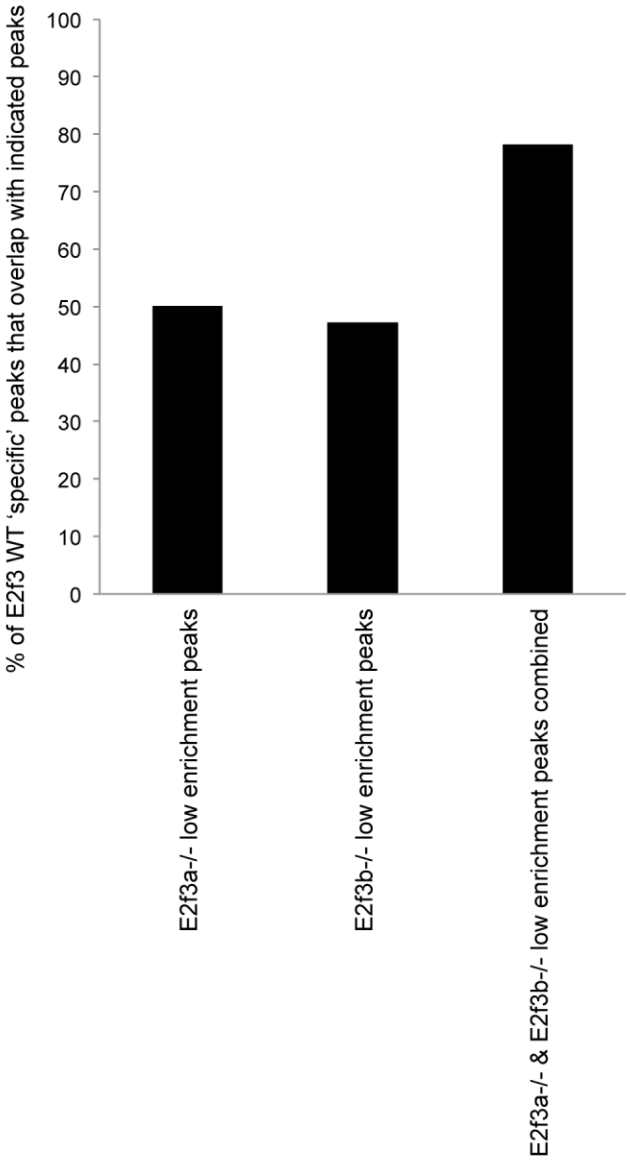


B



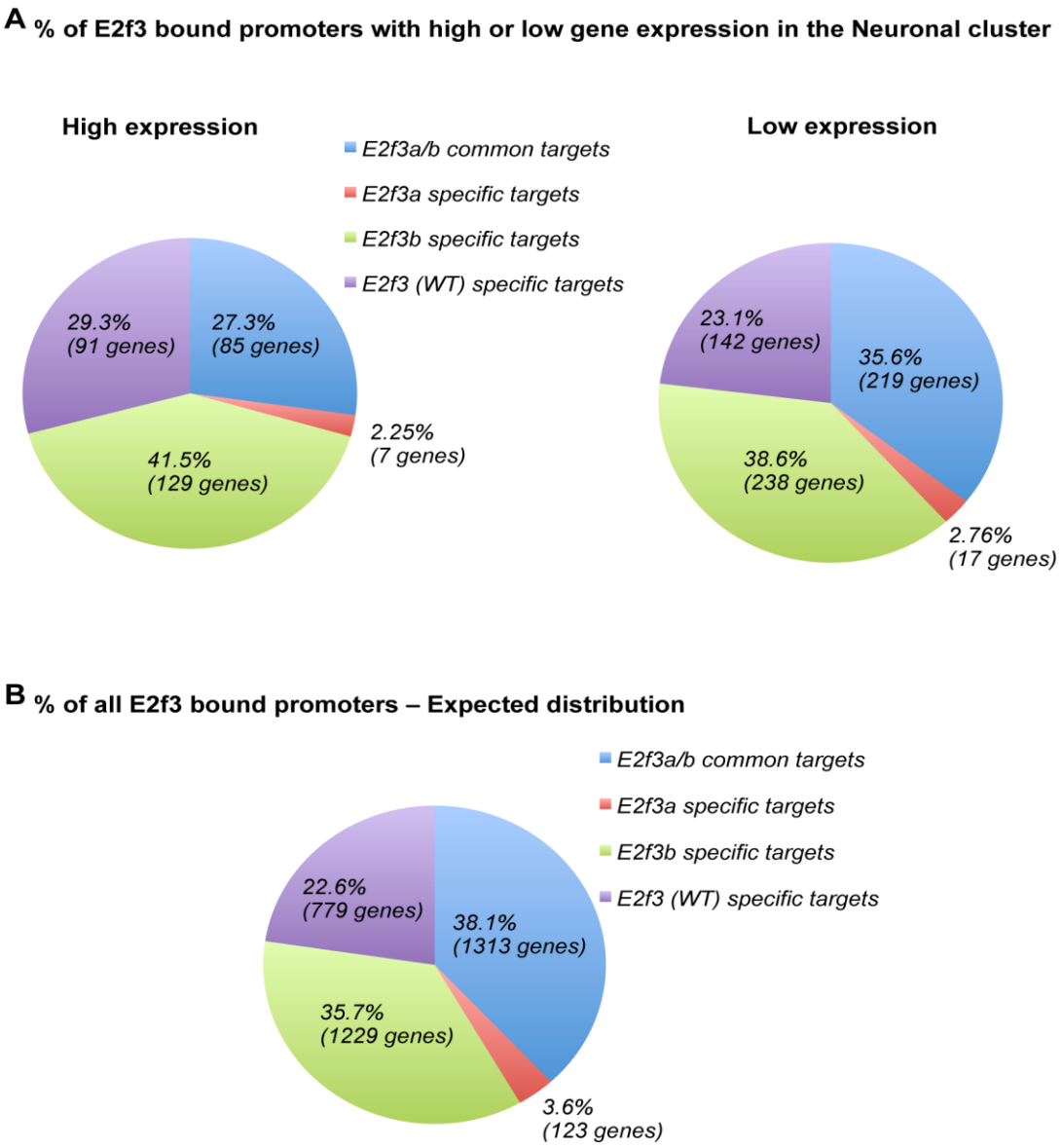
Supplemental Figure 3.1. Distribution of E2f3 and E2f4 bound loci relative to transcriptional start sites across mouse chromosome 7

ChIP-chip experiments were performed to determine E2f3 and E2f4 binding sites across the entire mouse chromosome 7. E2f bound loci were ranked according to their distance to the nearest TSS, and were binned into 500bp to 5kb regions. The percent of all E2f peaks within each bin range was calculated. The vast majority of loci bound by E2f3 (A) and E2f4 (B) are located within 3.5kb upstream and 1.5kb downstream of the nearest TSS.

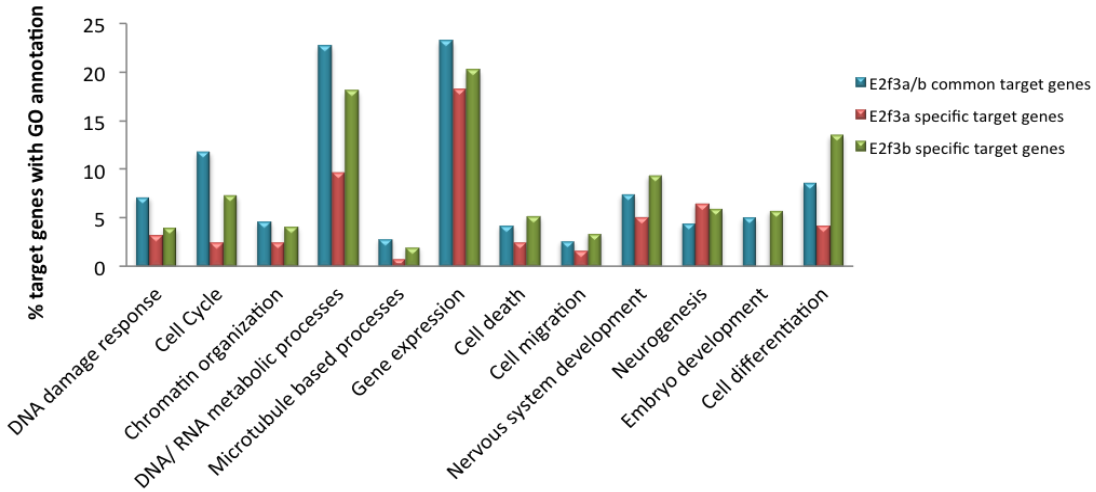


Supplemental Figure 3.2. Overlap between E2f3 peaks identified in wild-type cells only and E2f3a-/- and E2f3b-/- peaks below our FDR cut-off

The percentage of E2f3 peaks identified in wild-type (WT) cells only ('E2f3 'WT' specific targets) with an FDR rating of <0.1 that overlap with E2f3 peaks identified in E2f3a-/- or E2f3b-/- neurospheres that have an FDR rating of >0.1 ('low enrichment peaks'). In total. 78% of E2f3a-/- and E2f3b-/- low enrichment peaks overlapped with E2f3 WT 'specific' peaks.



Supplemental Figure 3.3. Distribution of E2f3 target genes highly expressed in neuronal tissues among different categories of E2f3a/b bound targets
(A) The lists of E2f3 targets belonging to the cluster of genes that were highly expressed (left-hand side) or showing low expression (right-hand side) in neuronal tissues were extracted. The percentage of these genes that were identified as targets specific to E2f3a or E2f3b, common to both E2f3a and E2f3b, or bound by E2f3 in wild-type cells only are shown, with the number of genes in each category shown in parentheses.
(B) For comparison with panel A, the percentage of E2f3 bound promoters belonging to specific E2f3 isoform categories are shown, with the number of genes in each category shown in parentheses. This figure demonstrates that the distribution of E2f3 target genes that are highly expressed or show reduced expression in neuronal tissues is similar to the distribution for all E2f3 targets: the majority are bound by E2f3b without close to half of these sites also bound by E2f3a; another 23-29% are bound by E2f3 in wild-type cells only.



Supplemental Figure 3.4. E2f3 isoforms control common cellular functions
GO analysis of E2f3a and E2f3b common and specific target genes, expressed as the percentage of all target genes in each group with a particular GO annotation (GO annotations indicated at the bottom of the graph). Target genes were identified as the gene whose TSS is closest to each E2f peak.

GO term	**E2f3a/b common genes** *% of genes (# of genes)*	**E2f3a specific genes** *% of genes (# of genes)*	**E2f3b specific genes** *% of genes (# of genes)*
DNA damage response	7.18% (99)	3.3% (4)	3.99% (49)
Cell cycle	11.83% (163)	2.5% (3)	7.32% (90)
Chromatin organization	4.64% (89)	2.5% (3)	4.15% (51)
DNA/ RNA metabolic processes	22.79% (314)	15.8% (19)	18.14% (223)
Microtubule based processes	2.9% (40)	0.8% (1)	2.03% (25)
Gene expression	23.29% (321)	18.3% (22)	20.26% (249)
Cell death	4.28% (59)	2.5% (3)	5.21% (64)
Cell migration	2.69% (37)	1.7% (2)	3.42% (42)
Nervous system development	7.47% (103)	5.1% (6)	9.44% (116)
Neurogenesis	4.42% (58)	6.5% (8)	5.94% (73)
Embryo development	5.08% (70)	0% (0)	5.78% (71)
Cell differentiation	8.6% (109)	4.2% (5)	13.59% (167)

Supplemental Table 3.1. Quantification of select gene ontology processes among genes targeted by E2f3a and E2f3b
Table displaying the percent and number (in parentheses) of genes bound by E2f3 in each group belonging to particular GO annotations, as indicated by GREAT and NIH-DAVID bioinformatics analysis.

CHAPTER 4

GENERAL DISCUSSION

4.1 Thesis Summary and Major Findings

The molecular processes underlying the generation of neurons in the brain are implemented in a well orchestrated, step-wise fashion, and cell cycle regulation has been intimately linked for some time to the proper regulation of neurogenesis during brain development (McConnell and Kaznowski, 1991). Understanding the mechanisms that control the generation of distinct cell fates during neurogenesis, and the contributions of cell cycle regulatory proteins to these processes, is important knowledge in the context of neurodevelopmental disorders, stem cell biology, and the design of neuronal replacement therapies in the injured and/or aging brain.

The central topic of this thesis is to determine the relative roles of the transcription factors E2F3a and E2F3b, two important cell cycle effector proteins, in the regulation of neural precursor cell fate decisions. Previous publications from our lab implicated the E2F3 transcription factor as a regulator of NPC proliferation in the embryonic and post-natal brain (Mcclellan et al., 2007; 2009), and in this dissertation we have demonstrated a much more extensive regulatory role for E2F3 in NPCs, highlighting it as a regulator of multiple cell fate decisions. Using knock-out mouse models of *E2f3a* and *E2f3b*, we have shown that in addition to proliferation E2f3 also impacts neural stem cell self-renewal and the capacity of NPCs to commit to a neuronal fate. These knock-out mouse models revealed that E2f3 isoforms function in a non-redundant, functionally opposing manner to balance maintenance of the NPC pool with neurogenesis, where loss of E2f3a leads to increased NSC self-renewal at the expense of neuronal differentiation, and loss of E2f3b results in increased differentiation and reduced NPC proliferation.

Stemming further from these findings, we identified the stem cell pluripotency gene *Sox2* as a direct target of E2f3 isoforms. Our lab has demonstrated previously that the pRB-E2F pathway can influence cell fate choices in the forebrain by controlling the expression of genes or pathways whose primarily role is unrelated to classical cell cycle regulation (Vanderluit et al., 2004; Ruzhynsky et al., 2007; Vanderluit et al., 2007b; Mcclellan et al., 2009; Andrusiak et al., 2011). Our discovery of *Sox2* as an E2f3 target further supports the hypothesis that E2F transcription factors can control cell cycle-independent processes through the regulation of non-classical target genes, and highlights E2F3 isoforms as direct regulators of neural stem cell fate decisions and pluripotency gene expression, with implications for other stem cell populations. Taken together, our findings have revealed both new cellular roles for E2F3 in stem cell self-renewal and neuronal commitment as well as an unexpected mechanism of E2F-dependent transcriptional regulation, where classical activator and repressor E2Fs function in unique roles.

We have also demonstrated in this dissertation, by identifying gene promoters bound by E2f3a, E2f3b and E2f4 on a genome-wide basis, that E2Fs are likely to influence diverse cell fate regulatory processes in NPCs. These processes include not only cell cycle regulation, but also cell death, differentiation, maintenance of the precursor state, neuronal commitment, and stem cell self-renewal. The target genes identified pertaining to these categories suggest that E2fs control not just single genes that are implicated in a given process or cellular pathway, but multiple core regulators of NPC fate decisions and regulatory pathways. Furthermore, the majority of these target promoters are bound by more than one E2f factor, and while it is likely that target genes other than *Sox2* are similarly regulated by different E2fs in an antagonistic manner, the relatively modest phenotypes of

individual *E2f* knock-out mice coupled with the large number of target genes we discovered suggests that considerable redundancy exists among E2fs at these loci. These findings suggest that the E2F family is an important regulator of diverse neural precursor cell fate processes by controlling the expression of a wide-range of previously unidentified target genes.

4.2 An Expanded Requirement for E2Fs in Neural Precursor Cell Fate Decisions

The existence of a link between cell cycle regulation and neural precursor cell fate decisions is well appreciated, as described in Chapter 1. However, the mechanisms by which these processes are related at the molecular level, and furthermore, whether cell cycle regulatory proteins themselves directly regulate genes that control diverse cell fate decisions, are largely unknown. A recent study demonstrated that changes in cell cycle dynamics (particularly the length of S phase) between expanding NPCs versus those committed to undergoing neurogenesis in the developing brain affects the expression of key genes involved not only in cell cycle control and DNA replication and repair, but also chromatin remodeling, neural precursor cell maintenance and neurogenesis (Arai et al., 2011b). One possibility for this result is that the cell cycle regulatory pathway, of which E2fs are the ultimate effectors, can directly regulate the expression of genes with divergent functions that impact neural precursor cell fate choices. As discussed above and in Chapter 1, our lab has discovered a small number of genes with direct, cell cycle independent roles in controlling NPC processes that are regulated by the pRB-E2F family (specifically the Notch/Hes and Shh pathways, as well as the *Fgf2*, *Dlx1*/*Dlx2*, and *Neogenin* genes (these publications appear in the supplement to this thesis)), supporting this possibility. Here, we have expanded upon these

findings significantly by: 1) discovering and functionally validating a new E2f target gene, *Sox2*, as a mediator of E2f3-dependent stem cell self-renewal, neuronal differentiation and, ultimately, cognitive function; and, 2) identifying as putative E2f targets an extensive number of genes whose protein products play key roles in the fundamental cell fate decisions that occur in neural precursor cells.

Our findings in Chapter 3 highlighted E2f3 isoforms as more wide-spread regulators of development and differentiation-related processes compared to E2f4, but interestingly, both E2f3 and E2f4 were found at the promoters of a number of core regulators of NPC regulatory processes; select examples include *Notch1*, *Ascl1*, *Hes5*, *Sox2*, *Numb*, *Pax6*, *Shh* and *Olig2*. Furthermore, as the majority of genes involved in neurogenesis and neurodevelopment are bound by more than one E2f factor (including E2f3a, E2f3b and E2f4), this suggests that E2F family members cooperate with one another to control the expression of key regulatory genes in NPCs. It is currently unknown if multiple E2F family members are able to bind simultaneously to a specific genomic region, or if this simply reflects heterogeneity of E2F binding among a population of cells or binding of different E2F factors to the same location at different times (ie. during different cell cycle phases). Regardless of E2F genomic binding dynamics, coordinate regulation of gene expression by E2F family members in NPCs could include binding of either multiple activator or repressor E2Fs to specific loci to regulate gene expression in a redundant or even additive manner, or the targeting of both activator and repressor E2Fs to promoter regions, resulting in antagonistic control of gene expression. Our data, demonstrating that E2f targets are bound by various combinations of E2f factors that are correlated with both transcriptional activation and repression, in fact suggests instead that a combination of each of these scenarios may

occur at E2F regulated loci. The data supporting these conclusions will be briefly discussed below.

Our analysis of *Sox2* regulation by E2f3 isoforms unexpectedly revealed a repressive function for E2f3a and a transcriptional activation function for E2f3b, suggesting that the transcriptional activity of E2f3 isoforms may be quite distinct in NPCs compared to other cell types. This was intriguing because E2f3a has previously been linked predominantly to gene activation in proliferating populations *in vivo* (Tsai et al., 2008; Chong et al., 2009b). Our ChIP-chip analysis was, however, not consistent with a pervasive role of E2f3a as a transcriptional repressor or with E2f3b as an activator, suggesting that this unique transcriptional activity observed at the *Sox2* locus is limited to specific loci. Future experiments focused on determining the effects of E2f3a and E2f3b on the expression levels of E2f3 target genes, either by analyzing select targets or performing broad-scale gene expression analysis in NPCs in which E2f3 isoforms are absent or are over-expressed, will help to reveal the extent of this phenomenon. Additionally, we presented data suggesting that p107 functions in a complex with E2f3a to repress *Sox2* expression (Chapter 2) in NPCs. We have previously demonstrated an important role for p107 in repressing the self-renewal capacity and proliferative expansion of NPCs, a role that is distinct from that of pRB (Ferguson et al., 2002; Vanderluit et al., 2004; Ferguson et al., 2005). Thus, a broad-scale examination of p107 genomic binding sites in NPCs will clarify the extent of E2f-mediated repression within this population, and will help to uncover any other target genes that may be regulated in an opposing fashion by E2f3 isoforms akin to *Sox2* regulation.

Analysis of our ChIP-chip data revealed, in fact, a wide-spread role for all E2fs examined in targeting the promoters of transcriptionally active genes. This phenomenon had

previously been described for E2F1 and E2F4 in a collection of cell lines (Xu et al., 2007), but had not been demonstrated in primary cell cultures, nor for E2f3 isoforms. Even more surprising was the fact that, although E2f3a-bound genes on average appear to have higher expression levels than E2f3b-bound targets, target genes that are either highly expressed or repressed specifically in neural tissues are similarly bound by different combinations of E2f3 isoforms. More specifically, approximately 40% of high or low expressing E2f3 target genes in neural tissues are bound by E2f3b only, while one quarter to one third of these genes are bound by both E2f3a and Ef23b or by E2f3 in WT cells only (only a small 2-3% are bound by E2f3a alone). While it is acknowledged that these genomic binding patterns provide correlative rather than absolute data regarding the effects of E2f3 isoform binding on gene expression levels, they nevertheless suggest that E2f3a and E2f3b, whether binding to common or isoform-specific target genes, are equally correlated with highly activated and repressed genes.

These binding patterns are similar to the pattern obtained for all E2f3 target genes in NPCs, which demonstrated that 36% of genes are bound by E2f3b alone, 3.6% by E2f3a alone, 38% by both E2f3a and Ef23b and finally, 23% are enriched for E2f3 only when both isoforms are present (in WT cells only), suggesting cooperative binding by E2f3a and E2f3b or the requirement for both to reach a threshold level of enrichment at these sites. This data, along with our finding that 73% of E2f3 target genes are also bound by E2f4, suggests that the transcriptional regulation of E2F target genes in NPCs is controlled by different combinations of E2F family members, often with multiple E2Fs targeting a specific site. This arrangement is likely the reason why phenotypes of single *E2f* knockout mice have not revealed this vast repertoire of target genes, and suggests that analysis of compound *E2f*

knockouts are more likely to reveal phenotypes and deregulation of target genes associated with neurogenesis and NPC fate decisions. Some identified E2f target genes have, however, been shown to exhibit changes in expression, correlating with phenotypic changes, following loss of single E2f proteins (*Sox2* and *Shh* (Chapter 2 and (Ruzhynsky et al., 2007)). Our work highlights the requirement for an analysis of forebrain phenotypes and target gene regulation in both single and compound *E2f* knockout mice to fully appreciate the role of individual E2Fs in controlling gene expression programs in NPCs. A further strength of our study in Chapter 3 is that we were able to identify the breadth of E2F activity in an actively expanding population of neural precursor cells in a WT setting, demonstrating for the first time that E2Fs are likely wide-spread regulators of multiple genes and pathways that impact cell fate related processes during neurogenesis.

4.3 Implications for E2Fs in Stem Cell Biology

Through our analysis of E2f3 and E2f4 target genes in NPCs (Chapter 3), and moreso our functional validation of *Sox2* as a target of E2f3 isoforms (Chapter 2), we have established the E2F family as an important regulator of neural stem cell self-renewal and the decision of NPCs to undergo differentiation or maintain a precursor state. Accumulating evidence over the past decade has implicated the pRB-E2F pathway in controlling the maintenance and behaviour of at least some stem cell populations in both plants and mammals, and loss of pRB function in select stem cell types is thought to alter their fate and give rise to cancer (Sage, 2012). For example, functional suppression of the single pocket protein in *Arabidopsis thaliana*, RBR, or overexpression of *E2Fa*, leads to a specific increase in the number of stem cells in the root meristem, and *RBR* over-expression causes these cells to

rapidly differentiate (Wildwater et al., 2005). *RBR* loss also results in an expanded stem cell pool and disrupts fate determination in the male germline (Chen et al., 2009c). This system revealed a role for the pRB-E2F pathway in the maintenance of the stem cell state. In a well characterized mammalian example, pRb is required specifically within trophoblast stem (TS) cells, but not within derivative progenitor cells, to control trophoblast proliferation and differentiation (Wenzel et al., 2007). *Rb1* loss in TS cells results in an increased TS cell pool, structural disruptions of the placenta, and embryonic death by E15.5. Furthermore, this critical function of pRb was shown to largely result from deregulation of *E2f3* within the TS cell pool. The pRB-E2F pathway has also been implicated in controlling the quiescent state, proliferative expansion, differentiation potential, and/ or the self-renewal capacity of retinal (Chen et al., 2004; MacPherson et al., 2004; Chen et al., 2009a; McEvoy et al., 2011), mesenchymal (Gutierrez et al., 2008), hematopoietic (Daria et al., 2008; Viatour and Sage, 2011), intestinal (Haigis et al., 2006; Yang and Hinds, 2007; Chong et al., 2009b), mammary gland (Jiang et al., 2010), epithelial (Ruiz et al., 2004), muscle (Huh et al., 2004; Hosoyama et al., 2011), liver (Viatour and Sage, 2011), and neural (Ferguson et al., 2002; Vanderluit et al., 2004; Fasano et al., 2007; Vanderluit et al., 2007b; Sutter et al., 2010) stem and progenitor cell populations. Despite these links, the mechanisms underlying pRB-E2F dependent regulation of stem cell populations remain poorly understood, and a better understanding of how this pathway influences stem cell fate decisions may enhance our potential to control stem cell-based functions for therapeutic purposes (Sage, 2012).

As transcription factors, E2Fs are the ultimate effectors of the pRB cell cycle regulatory pathway; therefore identifying target genes of E2Fs in stem and progenitor cell populations is a key step in understanding the mechanisms of cell cycle-dependent regulation

of stem cell fate decisions. Furthermore, since we have previously identified a role for E2f4 in NSC self-renewal, and we and others have identified a specific role for E2f3 in controlling the expansion of stem and progenitor populations (Mcclellan et al., 2007; Wenzel et al., 2007), these two E2F family members are particularly appropriate candidates for study in this context. Our work therefore provides valuable information regarding the mechanisms through which cell cycle regulation can impact precursor cell fate decisions. It will be important for future studies to assess the effects of E2F deregulation on the expression of these target genes in neural precursor cells as well as precursors derived from other tissues, and to identify E2F target genes in other primary stem and progenitor cell populations. This will allow us to understand which E2F-dependent regulatory mechanisms are conserved among all precursor cell types and which may be unique to certain tissues. Interestingly, our analysis of E2f3 genomic binding patterns in neural versus muscle precursor cells unexpectedly revealed extensive tissue-specificity of E2f3-dependent target genes between these two cell types, where cell cycle regulatory genes are conserved, but genes related to developmental processes are unique to each cell type. This clear tissue-specificity has not been observed for other E2F family members, and our observation raises the possibility that E2F3 may uniquely regulate distinct target genes or even regulatory mechanisms in different precursor cell populations. Future studies should evaluate the target genes of E2F3 isoforms in more diverse stem and progenitor cell populations to clarify this issue.

Perhaps not surprisingly, the contributions of the pRB-E2F pathway to regulation of the pluripotent state of embryonic stem cells (ESCs) and in facilitating reprogramming in the generation of induced pluripotent stem (iPS) cells have recently begun to be explored. Currently, there is very little known regarding the potential role of pRB-E2F in regulating

pluripotency, but initial evidence suggests that this pathway plays an active role in this process. For example, sh-RNA molecules targeted against *RB1* promoted somatic cell reprogramming (Samavarchi-Tehrani et al., 2010), and pRB is known to be targeted for inactivation by caspases during the reprogramming process (Li et al., 2010). Furthermore, a recent study demonstrated that homeostatic levels of the pocket protein family are required in human ESCs for their survival and to maintain them in a self-renewing, proliferative state (Conklin et al., 2012). Our study identifying the pluripotency factor *Sox2* as a target gene of E2f3 in NPCs (Chapter 2) further suggests that the pRB-E2F pathway may be an important regulator of the pluripotent stem cell state, and provides significant mechanistic insight into how this regulation may occur. Two other studies published in parallel to ours also highlighted the importance of *Sox2* regulation in different stem cell populations by cell cycle regulatory proteins: p21 is required to repress *Sox2* in adult NSCs to prevent their exhaustion (Marqués-Torrejón et al., 2013), and p27, in collaboration with p130 and E2F4, is important for the repression of *Sox2* during embryonic stem cell differentiation (Li et al., 2012). An important focus for future studies would be to determine if E2f3 regulates *Sox2* expression in other tissue-specific and pluripotent stem cell populations, or if E2f3 functions in this capacity specifically in NPCs. We are also interested in understanding the mechanisms that lead to permanent silencing of genes that promote the stem cell state, like *Sox2*, following cell cycle exit and neuronal differentiation in the brain, and we hypothesize that E2F4 facilitates the silencing of such genes by recruiting p130 to form active repressor complexes in post-mitotic neurons. Analysis of these important questions will further clarify the mechanisms by which cell cycle regulatory proteins control the decision between stem cell maintenance and differentiation in the brain, as well as other tissues and cell types.

4.4 Conclusions

The studies herein have presented evidence suggesting that the E2F family of transcription factors are important regulators of genes that control the potential of neural stem cells to adopt distinct cell fates. Furthermore, we have identified E2F3 isoforms as important regulators of the balance between self-renewal and differentiation in neural precursors, and have identified the pluripotency gene *Sox2* as a regulatory target in this context, linking E2Fs to pluripotent gene regulation. Our work has revealed important novel functions for the E2F family, highlighting mechanisms by which the cell cycle regulatory pathway is linked to the control of NPC fate determination, and has unexpectedly revealed E2F3 as a regulator of tissue-specific transcriptional programs. These results support the hypotheses that E2F transcription factors control a variety of cellular functions by regulating the expression of cell cycle-independent target genes and that E2F3 isoforms along with E2F4 are wide-spread regulators of cell fate related processes in NPCs, revealing a much more expansive regulatory role for E2Fs than previously appreciated.

CHAPTER 5

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APPENDIX A

Unpublished Supplemental Data

1 Figure

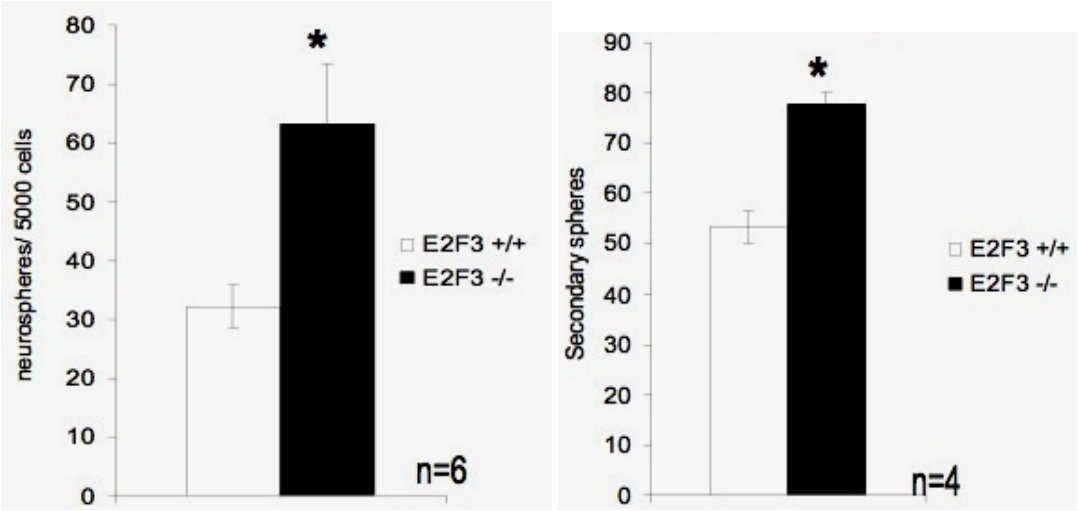

Figure A1. Loss of E2f3 leads to increased neurosphere self-renewal.

GE tissue was dissected from E2f3+/+ and E2f3-/- littermates at day E14.5 of gestation. Cells were plated in stem cell medium (SCM) containing FGF at a density of 10 cells/ ul. The number of primary spheres of 100um width or greater that formed after 7 days was quantified. Spheres measuring 150um +/- 10um were individually plucked from primary sphere cultures, triturated to a single cell suspension and plated in SCM with FGF. The number of secondary spheres was quantified after 7 days in culture. This data demonstrates that E2f3-/- NPCs derived from the GE have an enhanced self-renewal potential compared to E2f3+/+ precursors.

APPENDIX B

Curriculum Vitae

Lisa Julian

EDUCATION

Degree in progress:

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

Degrees held:

M.Sc. 2006	Biochemistry University of Western Ontario London, Ontario, Canada Thesis Supervisor: Dr. Fred Dick
B.Sc. 2003	Honours Bachelor of Medical Sciences, Biochemistry University of Western Ontario London, Ontario, Canada Thesis Supervisor: Dr. Eric Ball

WORK EXPERIENCE

2006-present	Graduate student (Ph.D.), Department of Cellular and Molecular Medicine, Neuroscience program, University of Ottawa
2006-2009	Exam proctor, Faculty of Medicine, University of Ottawa
May-Aug. 2006	Research Assistant, London Regional Cancer Program, Department of Oncology, University of Western Ontario
2003-2006	Graduate student (M.Sc.), Department of Biochemistry, University of Western Ontario
2002-2003	Honour's thesis project, Department of Biochemistry, Universty of Western Ontario
2002-2003	Exam proctor, Faculty of Medicine and Dentistry, University of Western Ontario
May-Aug. 2003	Summer Research Student, Department of Biochemistry, University of Western Ontario
May-Aug. 2002	Summer Research Student, Department of Biochemistry, University of Western Ontario

2001-2002 Undergraduate research volunteer student, Department of Biochemistry, University of Western Ontario

SCHOLARLY AND PROFESSIONAL ACTIVITIES

2008-2010 President, Cellular and Molecular Medicine/ Neuroscience (CMM/NSC) Student's Council (University of Ottawa)

2007-2008 Senior VP Academics, CMM/NSC Student's Council (University of Ottawa)

2007-2010 Graduate Student Representative, CMM/NSC Monthly Departmental Faculty Meetings (University of Ottawa)

2007-2010 Graduate Student Representative, Faculty of Medicine Graduate Studies Committee (University of Ottawa)

2006-2010 CMM/NSC Student's Council Member (University of Ottawa)

2003-2005 Science Instructor and Demonstrator, Let's Talk Science School Partnership Program (University of Western Ontario)

2002-2003 Biochemistry Student's Council Member (University of Western Ontario)

2002-2003 Undergraduate Student Representative, Biochemistry Faculty Academic Committee (University of Western Ontario)

2000-2001 Science Activity Demonstrator, Let's Talk Science (University of Western Ontario)

AWARDS AND SCHOLARSHIPS

2008-2011 CIHR Frederick Banting and Charles Best Canada Graduate Scholarship – Doctoral Award: $35 000 annually

2008-2009 Ontario Graduate Scholarship (declined): $15 000

2007-2008 Ontario Graduate Scholarship: $15 000

2007 Stem Cell Network Trainee Award, Advanced Multi-Colour Flow Cytometry and Cell Sorting Workshop, Vancouver B.C.

2007-2008 Ontario Graduate Scholarship in Science and Technology (declined): $15 000

2006-2007 Ontario Graduate Scholarship in Science and Technology: $15 000

2006-2012 Award of Excellence, University of Ottawa: Tuition fees

2006 Conference Travel Award, Division of Experimental Oncology,

UWO: $1 000

2003-2005 CIHR/UWO Strategic Training Initiative in Cancer Research and Technology Transfer (including funds from the Southwestern Ontario Women's Charity Cashspiel): $22 600 annually

2003-2005 Special University Scholarship, University of Western Ontario $5 600 annually

2002 Summer Research Studentship, University of Western Ontario: $4 800

2001-2003 Dean's Honour List, University of Western Ontario

1999-2000 Western Scholarship of Excellence, University of Western Ontario: $2 000

PUBLICATIONS

Papers in refereed journals	7
Papers in preparation	2
Select meeting Abstracts	17
Invited presentations	1

Papers in Refereed Journals:

1. **Lisa M. Julian**, Renaud Vandenbosch, Catherine A. Pakenham, Matthew G. Andrusiak, Angela P. Nguyen, Kelly A. McClellan, Devon S. Svoboda, Diane C. Lagace, David S. Park, Gustavo Leone, Alexandre Blais, and Ruth S. Slack. Opposing regulation of *Sox2* by cell cycle effectors E2f3a and E2f3b in neural stem cells. *Cell Stem Cell* (2013). 12(4): 440-452.

2. Noel Ghanem, Matthew G. Andrusiak, Devon Svoboda, Sawsan M. Al Lafi, **Lisa M. Julian**, Kelly A. McClellan, Yves De Repentigny, Rashmi Kothary, Marc Ekker, Alexandre Blais, David S. Park, and Ruth S. Slack. The Rb/E2F pathway modulates neurogenesis through direct regulation of the *Dlx1/Dlx2* bigene cluster. *Journal of Neuroscience* (2012). 32(24): 8219-8230.

3. Andrusiak MG, McClellan KA, Dugal-Tessier D, **Julian LM**, Rodrigues SP, Park DS, and Slack RS. Rb/E2F regulates expression of neogenin during neuronal migration. *Molecular and Cellular Biology* (2010). 31(2): 238-247.

4. McClellan KA, Vanderuit JL, **Julian LM**, Andrusiak MG, Dugal-Tessier D, Park DS, and Slack RS. The p107/E2F pathway regulates fibroblast growth factor 2 responsiveness in neural precursor cells. *Molecular and Cellular Biology* (2009). 29(17): 4701-13.

5. Seifried LA, Talluri S, Cecchini M, **Julian LM**, Mymryk JS, and Dick FA. pRB-

E2F1 complexes are resistant to adenovirus E1A-mediated disruption. *Journal of Virology* (2008). 29(17): 4701-13.

6. **L.M. Julian,** O. Palander, L.A. Seifried, J.E.G. Foster, and F.A. Dick. Characterization of an E2F1-specific binding domain in pRB and its implications for apoptotic regulation. *Oncogene* (2008). 27(11):1572-1579.

7. Isaac, C.E., Francis, S.M., Martens, A.L., **Julian, L.M.,** Seifried, L.A., Erdmann, N., Binne, U.K., Harrington, L., Sicinski, P., Berube, N.G., Dyson, N.J., and Dick, F. A. The retinoblastoma protein regulates pericentric heterochromatin. *Molecular and Cellular Biology* (2006). 26 (9): 3659-71.

Papers in Preparation:

1. **Julian LM**, Liu Y, Pakenham CA, Leone G, Slack RS, and Blais A. Tissue-specific binding of E2f3 isoforms to promoters of diverse cell fate regulatory genes in neural precursor cells.

2. **Julian LM**, Dugal-Tessier D, Blais A, and Slack RS. E2f4 regulates Shh signaling in the ventral telencephalon via transcriptional control of *Shh* and *Gli3*.

Meeting Abstracts

1. * **Lisa Julian**, Catherine Pakenham, Renaud Vandenbosch, Kelly McClellan, David Park, Gustavo Leone, Alexandre Blais, and Ruth Slack. E2f3 isoforms control neurogenesis by regulating *Sox2* expression. 2nd International Rb Meeting. November 2011. Toronto, Ontario. ***Invited speaker – oral presentation.**

2. **Lisa Julian**, Catherine Pakenham, Renaud Vandenbosch, Kelly McClellan, Yubing Liu, David Park, Gustavo Leone, Alexandre Blais, and Ruth Slack. E2f3 transcription factor isoforms differentially regulate precursor vs neurogenic states in the developing cortex to ensure proper neuronal output. International conference on Wiring the Brain: Making Connections. April 2011. Powerscourt, Co. Wicklow, Ireland. (Abstract selected for poster presentation).

3. Devon Svoboda, **Lisa M Julian**, Julien Sage, David S Park, and Ruth S Slack. Role of the Rb family proteins in neuronal differentiation and synaptogenesis. International conference on Wiring the Brain: Making Connections. April 2011. Powerscourt, Co. Wicklow, Ireland. (Abstract selected for poster presentation).

4. **Lisa Julian**, Catherine Pakenham, Renaud Vandenbosch, Kelly McClellan, Yubing Liu, David Park, Gustavo Leone, Alexandre Blais, and Ruth Slack. E2f3 isoforms balance precursor vs neurogenic states and bind a network of neurogenic gene promoters. 5th Annual Canadian Neuroscience Meeting. Quebec City, Canada. May 2011. (Abstract selected for poster presentation).

5. Catherine Pakenham, **Lisa Julian**, David Park, Gustavo Leone, and Ruth Slack. E2f3 regulates neural stem cell populations by modulating Polycomb group proteins. Estoril, Portugal. May 2011. (Abstract selected for poster presentation).

6. **Lisa Julian**, Catherine Pakenham, Renaud Vandenbosch, Vladimir Ruzhynsky, Yubing Liu, David Park, Gustavo Leone, Alexandre Blais, and Ruth Slack. Differential regulation of neural stem cell self-renewal and progenitor proliferation by distinct E2f3 isoforms: a genome-wide analysis. 18th Biennial Meeting of the International Society for Developmental Neuroscience. Estoril, Portugal. June 2010. (Abstract selected for poster presentation).

7. Catherine Pakenham, **Lisa Julian**, David Park, Gusravo Leone, and Ruth Slack. Maintenance of neural stem cell self-renewal by E2f3. 18th Biennial Meeting of the International Society for Developmental Neuroscience. Estoril, Portugal. June 2010. (Abstract selected for poster presentation).

8. **Lisa Julian**, Catherine Pakenham, Renaud Vandenbosch, Vladimir Ruzhynsky, Yubing Liu, David Park, Gustavo Leone, Alexandre Blais, and Ruth Slack. Differential regulation of neural stem cell self-renewal and progenitor proliferation by distinct E2f3 isoforms. First Annual International Rb Conference. Toronto, Ontario. November 2009. (Abstract selected for poster presentation).

9. **Lisa Julian**, Kelly McClellan, Catherine Pakenham, David Park, Gustavo Leone, and Ruth Slack. Individual E2f3 isoforms uniquely regulate neural precursor proliferation and self renewal. Cold Spring Harbor Laboratory/ Wellcome Trust: Mouse Genetics and Genomics – Development and Disease. Hinxton, United Kingdom. September 2009. (Abstract selected for poster presentation).

10. **Lisa Julian**, Kelly McClellan, Catherine Pakenham, David Park, Gustavo Leone, and Ruth Slack. Individual E2f3 isoforms uniquely regulate neural precursor proliferation and self renewal. Keystone Conferences: Deregulation of Transcription in Cancer. Killarney, Ireland. July 2009. (Abstract selected for poster presentation).

11. **Lisa Julian**, Kelly McClellan, Catherine Pakenham, David Park, Gustavo Leone, and Ruth Slack. E2f3 isoforms differentially regulate neural precursor expansion and stem cell self-renewal. Cold Spring Harbor – Mouse Genetics and Genomics. Cold Spring Harbor, New York. October 2008. (Abstract selected for poster presentation).

12. **Julian, L.M.,** McClellan, K.A., Park, D.S., Leone, G.W., and Slack, R.S. Regulation of Neural Progenitor Expansion and Stem Cell Self Renewal by Distinct Isoforms of the E2f3 Transcription Factor. Society for Neuroscience 37th Annual Meeting, San Diego, California, November 2007. (Abstract selected for poster presentation)

13. **Julian, L.M.,** McClellan, K.A., Park, D.S., Leone, G.W., and Slack, R.S. Regulation of Neural Progenitor Expansion and Stem Cell Self Renewal by Distinct Isoforms of the E2f3 Transcription Factor. Stem Cell Network 2007 Annual General Meeting, November 2007. (Abstract selected for poster presentation)

14. **Julian, L.M.** and Dick, F.A. An E2F1-'Specific' Binding Domain Within pRB is Required for Regulation of E2F1-Induced Apoptosis. The University of Western Ontario, Department of Oncology, *3rd Annual Research and Education Day*, London, Ontario, June 2006. (Abstract selected for poster presentation)

15. **Julian, L.M.** and Dick, F.A. An E2F1-'Specific' Binding Domain Within pRB

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APPENDIX C

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APPENDIX D

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Cell Stem Cell
Article

Opposing Regulation of *Sox2* by Cell-Cycle Effectors E2f3a and E2f3b in Neural Stem Cells

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SUMMARY

The mechanisms through which cell-cycle control and cell-fate decisions are coordinated in proliferating stem cell populations are largely unknown. Here, we show that E2f3 isoforms, which control cell-cycle progression in cooperation with the retinoblastoma protein (pRb), have critical effects during developmental and adult neurogenesis. Loss of either E2f3 isoform disrupts *Sox2* gene regulation and the balance between precursor maintenance and differentiation in the developing cortex. Both isoforms target the *Sox2* locus to maintain baseline levels of *Sox2* expression but antagonistically regulate Sox2 levels to instruct fate choices. E2f3-mediated regulation of *Sox2* and precursor cell fate extends to the adult brain, where E2f3a loss results in defects in hippocampal neurogenesis and memory formation. Our results demonstrate a mechanism by which E2f3a and E2f3b differentially regulate *Sox2* dosage in neural precursors, a finding that may have broad implications for the regulation of diverse stem cell populations.

INTRODUCTION

Stem cell-fate decisions, such as self-renewal, precursor cell maintenance, and commitment to differentiation have critical outcomes for embryonic development, tissue maintenance, tumor suppression, and regeneration. Cortical development depends on a precisely regulated balance of self-renewal within stem cell-like apical precursors (APs), production of rapidly proliferating basal progenitors (BPs), and differentiation of postmitotic neurons (Englund et al., 2005; Farkas and Huttner, 2008; Hutton and Pevny, 2011) (Figure 1A). Identifying mechanisms that control this balance can inform our understanding of developmental neurogenesis and, more broadly, reveal stem cell biological principles extending to embryonic stem cell differentiation, tumor formation, and tissue regeneration.

The pluripotency factor Sox2 is an established regulator of neural precursor proliferation, self-renewal, and differentiation during development and is also required for maintenance of adult stem cell populations in many different tissues (reviewed in Sarkar and Hochedlinger, 2013). Overexpression of Sox2 in both mouse and chick embryonic neural precursor cells (NPCs) results in maintenance of the $Sox2^+$ population and defective neurogenesis (Bani-Yaghoub et al., 2006; Graham et al., 2003). Conversely, loss of function of Sox2 in neural precursors leads to precursor loss and reduced or aberrant differentiation, depending on the tissue type and degree of Sox2 loss (Cavallaro et al., 2008; Favaro et al., 2009; Ferri et al., 2004; Graham et al., 2003; Miyagi et al., 2008; Taranova et al., 2006). Taken together, these studies reveal that the function of Sox2 is strongly influenced by dosage; thus, fine-tuning of transcription from the *Sox2* locus is crucial for the generation of the correct proportion of precursors versus differentiated cell types. Interestingly, a recent study finds that the Cyclin-dependent kinase inhibitor 1A (p21) binds a *Sox2* enhancer region to regulate Sox2 expression and adult neurogenesis, linking cell-cycle regulation with Sox2-mediated control of neural stem cell (NSC) expansion (Marqués-Torrejón et al., 2013).

Previous evidence suggests that the cell cycle machinery plays a key role in regulating the proliferative expansion and self-renewal capacity of NPCs (Nishino et al., 2008; Ruzhynsky et al., 2007; Vanderluit et al., 2004). However, how specific cell-cycle regulatory proteins function in this context remains poorly defined. The retinoblastoma pocket protein (pRb) family controls cell-cycle progression by binding and inhibiting the E2f family of transcription factors. E2fs are classified into the "activator" subclass, which drives proliferation and transcription, and the "repressor" subclass, the members of which are thought to repress gene transcription by modifying chromatin structure through association with pocket proteins (Asp et al., 2009). Earlier work has reported that E2f3 is the most highly expressed E2f family member in wild-type and pRb-deficient neural precursors (Callaghan et al., 1999), suggesting that it may be an important regulator of NPC functions. Understanding how the *E2f3* gene functions to regulate the cell cycle is not entirely straightforward, because the two isoforms (*E2f3a* and *E2f3b*) expressed from its locus have identical domains important for DNA binding, transactivation, and pocket-protein binding, and only their N termini are unique. Mice lacking both

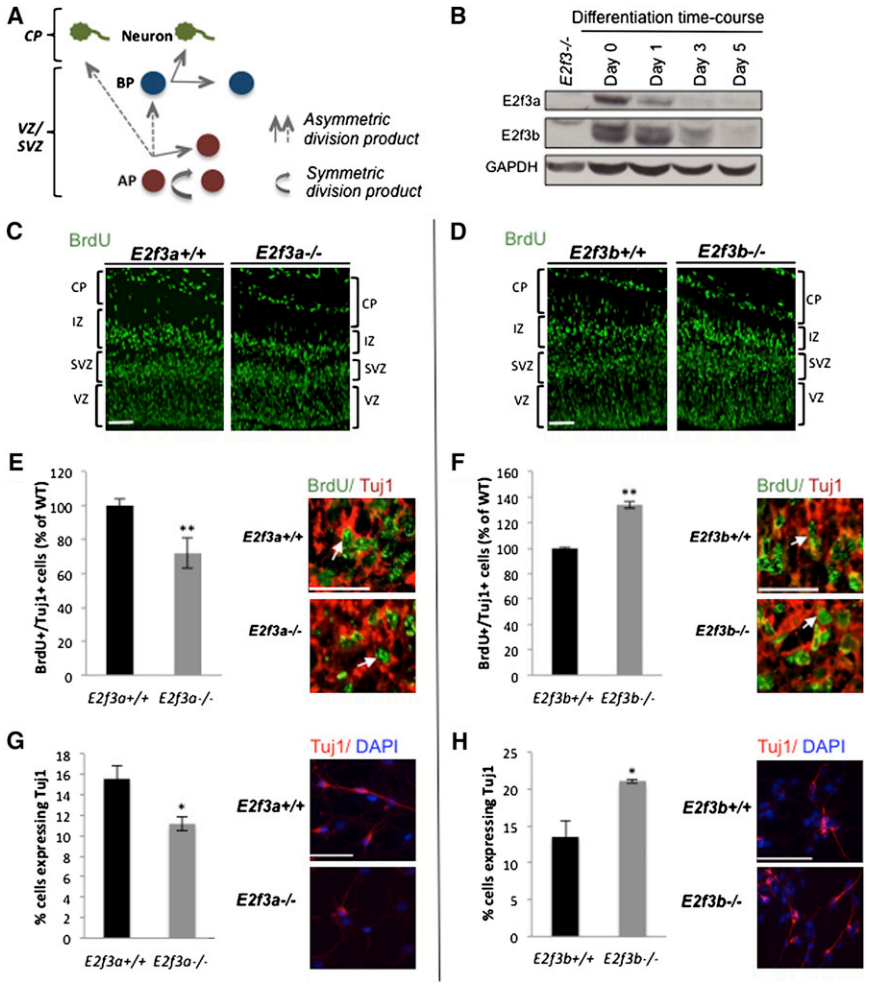

Figure 1. E2f3 Isoforms Are Differentially Required for Neuronal Commitment

(A) Cortical development depends on a finely controlled balance of AP cell proliferation, self-renewal, and differentiation. APs can divide symmetrically to expand their population or asymmetrically to generate one AP and a neuron, glial cell, or BP. BPs generate neurons through asymmetric divisions. CP, cortical plate.

(B) Immunoblot for E2f3 in cultured neurospheres induced to differentiate over 5 days. Both E2f3a and E2f3b are expressed in proliferating neurospheres (day 0), but expression is decreased as differentiation progresses (days 1, 3, and 5). GAPDH was included as a protein loading control.

(C and D) BrdU staining in E14.5 coronal sections following a 24 hr BrdU pulse to identify cells that have exited the cell cycle. Fewer BrdU$^+$ cells are observed in the *E2f3a*$^{-/-}$ SVZ and IZ, whereas more BrdU$^+$ cells are apparent in *E2f3b*$^{-/-}$.

(E and F) Sections described in (C) and (D) were immunostained for BrdU and Tuj1. The number of cells expressing both BrdU and Tuj1 was quantified within a defined area in the SVZ and IZ (arrows identify examples of quantified cells). Results are expressed as a percentage of *E2f3a*$^{+/+}$ average values ± SEM (n = 4).

(G and H) Neurospheres were expanded in vitro and, upon first passage, were cultured in differentiation media on poly-L-ornithine-coated dishes for 3 days, PFA fixed, and immunostained for Tuj1 and DAPI. *E2f3a*$^{-/-}$ possesses fewer Tuj1$^+$ cells; *E2f3b*$^{-/-}$ has more Tuj1$^+$ cells. Results are presented as the percentage of DAPI$^+$ cells expressing Tuj1 ± SEM (n = 4).

For (E)–(H): *p < 0.05, **p < 0.01. Scale bars represent 50 μm. See also Figure S1.

isoforms die perinatally due to cardiac defects (King et al., 2008), whereas those deficient in either isoform are fully viable (Danielian et al., 2008; Tsai et al., 2008), suggesting functional overlap. Tissue- and cell-type-specific analysis of pRb and E2f knockout mice suggests that E2f3a is generally a potent activator of transcription and proliferation, whereas E2f3b induces proliferation weakly and promotes differentiation (Asp et al., 2009; Chong et al., 2009; Danielian et al., 2008), but whether individual E2f3 isoforms make a distinct contribution to developmental and adult neurogenesis is currently unknown.

Here, we use mouse models deficient for either E2f3 isoform to reveal that E2f3a and E2f3b antagonistically regulate Sox2 expression in NSCs. In E2f3b-null animals, where E2f3a is the dominant isoform, we find that E2f3a represses *Sox2* in cooperation with the pRb family member p107, reduces precursor self-renewal, and promotes differentiation. Conversely, in E2f3a-null animals, where E2f3b is the dominant isoform, we find that E2f3b activates *Sox2* expression by recruiting RNA Polymerase II to its promoter, which leads to increased self-renewal and precursor expansion at the expense of differentiation. Knockdown of *Sox2* in E2f3a-deficient NPCs restored basal levels of self-renewal. Importantly, we find that adult E2f3a-null mice have impaired neurogenesis and a reduced capacity for hippocampal-dependent contextual learning, underscoring how the antagonism between E2f3 isoforms is conserved to regulate adult neurogenesis and affect memory formation.

RESULTS

E2f3 Isoforms Are Expressed in NPCs

E2f3 is a potent cell-cycle regulator and a highly expressed E2f family member in NPCs (Callaghan et al., 1999; McClellan et al., 2007), suggesting a potential role for E2f3 in this cell type. Interestingly, we observed that expression of both E2f3 isoforms is enriched in NPCs but reaches negligible levels by day 5 of differentiation in vitro (Figure 1B; Figures S1A and S1B available online), pointing to a regulatory role for both isoforms within the proliferating precursor pool. We asked whether E2f3 isoforms play an important role in regulating neural stem and progenitor cell-fate decisions by examining mouse lines deficient for *E2f3a* and *E2f3b* (Chen et al., 2007; Tsai et al., 2008).

E2f3a and E2f3b Deficiency Impacts NPC-Fate Decisions in an Opposing Manner

We first asked whether loss of E2f3 isoforms impacts NPC-fate decisions by performing a neuronal commitment assay. Mice

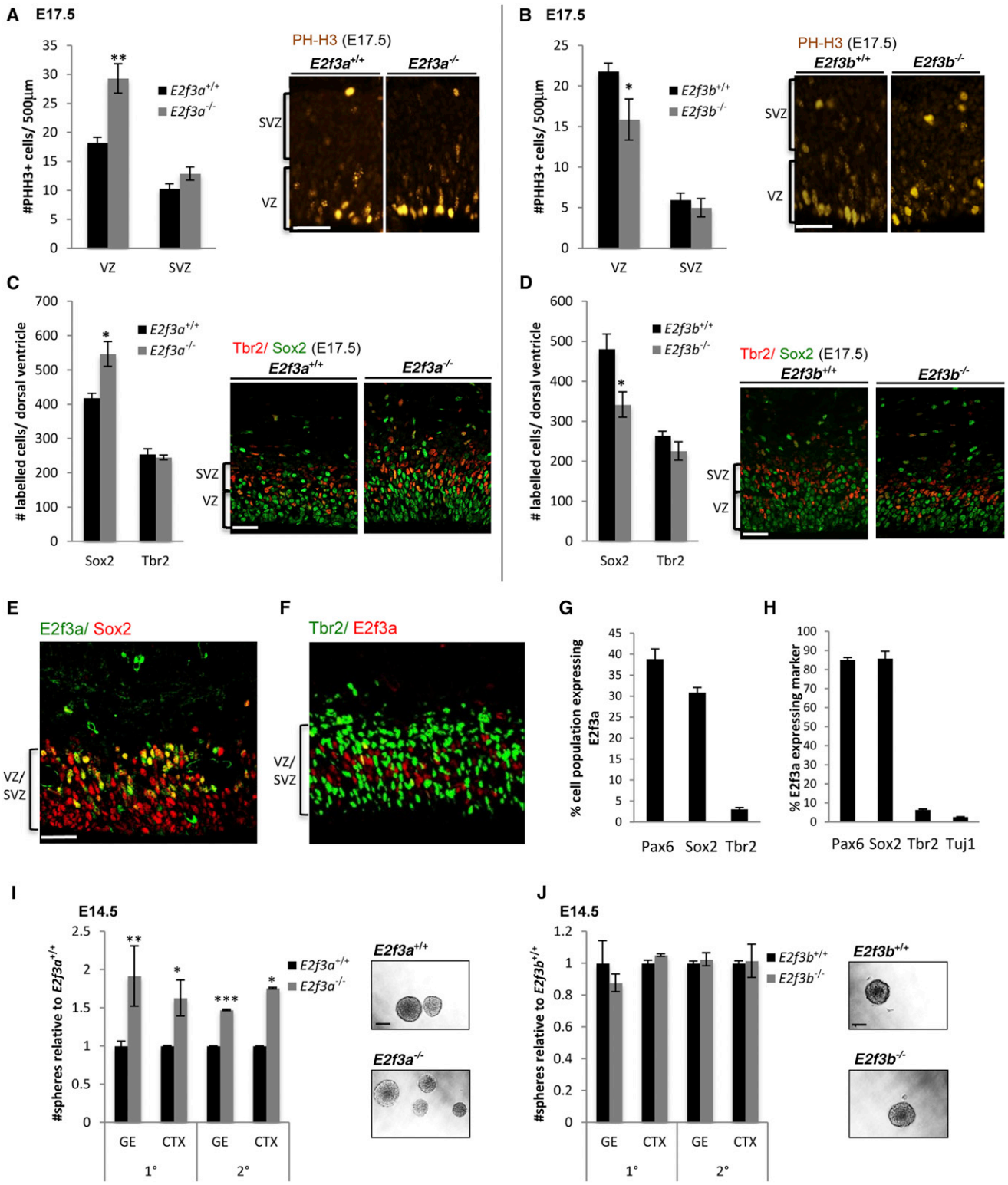

Figure 2. E2f3 Isoforms are Differentially Required for Regulation of NPC Numbers and Self-Renewal

(A and B) PH-H3 staining in E17.5 coronal sections to label mitotic cells. PH-H3$^+$ cells were quantified along the dorsal surface of the lateral ventricle in either the VZ or SVZ, and numbers were normalized to a defined ventricular length (500 µm). Quantification demonstrates an expansion in *E2f3a*$^{-/-}$ and a decrease in *E2f3b*$^{-/-}$, specifically in the VZ (n = 4).

(C and D) Quantification of Sox2$^+$ and Tbr2$^+$ cells within the dorsal cortex at E17.5 demonstrates an increased number of Sox2$^+$ cells in *E2f3a*$^{-/-}$ and fewer Sox2$^+$ cells in *E2f3b*$^{-/-}$ (n = 4).

(legend continued on next page)

were given a single bromodeoxyuridine (BrdU) injection and were sacrificed 24 hr later for the purpose of visualizing $BrdU^+$ cells that had exited the cell cycle and initiated differentiation. There were visibly fewer $BrdU^+$ cells migrating into the subventricular zone (SVZ) and intermediate zone (IZ) of *E2f3a*$^{-/-}$ mice, but more $BrdU^+$ cells in these regions in E2f3b knockouts (Figures 1C and 1D), suggesting a differential commitment to neurogenesis. Newly committed cells that have undergone terminal mitosis can be identified by double labeling with BrdU and differentiation markers, including βIII-tubulin (TuJ1) and Doublecortin (DCX). These BrdU-positive cells are also negative for the proliferation marker Ki67. *E2f3a*$^{-/-}$ mice exhibited a significant reduction in newly committed cells that colabeled for BrdU and Tuj1 (Figure 1E) or BrdU and DCX (Figure S1C) and cells that were negative for Ki67 ($BrdU^+$/$Ki67^-$) (Figure S1D). In contrast, these same experiments revealed that E2f3b-deficient brains contain significantly more committed cells (Figure 1F; Figures S1E and S1F). These results were further supported in vitro by quantification of newly committed cells in neurosphere cultures induced to differentiate. Here again, E2f3a-deficient NPCs exhibited a reduction in differentiation, whereas E2f3b-deficient precursors had an increase in differentiating cells (Figures 1G and 1H). Deficiency of either E2f3 isoform does not lead to compensatory expression changes of other pRb and E2f family members (Figures S1G–S1J), demonstrating the specificity of E2f3-isoform-dependent phenotypes. Thus, deficiency of E2f3 isoforms impacts NPC-fate decisions in distinct ways: E2f3a loss reduces, whereas E2f3b deficiency increases, commitment to a neuronal fate.

Second, to determine whether E2f3 isoforms are similarly required to regulate the size of the neural precursor pool in an opposing manner, we quantified the number of proliferating NPCs during forebrain development by performing a 2 hr BrdU incorporation (S phase) and phosphohistone H3 (PH-H3) immunostaining (M phase). E2f3a loss resulted in an expanded neural precursor pool (Figures S2A and S2C), specifically affecting the $Sox2^+$ stem-like APs in the ventricular zone (VZ) (Figures 2A and 2C; Figure S2E) and culminating in a 38% increase in the size of this population by embryonic day 17.5 (E17.5) (Figure 2A). Loss of E2f3b resulted in an average 25% decrease in precursor numbers throughout development (Figures S2B and S2D), again specifically affecting Sox2-expressing stem-like APs (Figures 2B and 2D; Figure S2F). Concomitant with the expanded precursor population in *E2f3a*$^{-/-}$ brains, the neuronal output at birth was significantly reduced (e.g., a 24% decrease in later-born neurons, layers I–III) (Figure S3A). In contrast, neuronal output was increased in E2f3b knockouts (Figure S3B). Thus, E2f3a and E2f3b are differentially required for the regulation of both the expansion of the AP population and the commitment of AP cells to a neuronal fate.

E2f3 Is Expressed in Stem-like $Sox2^+$ APs

The impact of E2f3 isoforms on cell-fate decisions within the AP population suggests that E2f3 is expressed in $Sox2^+$ precursors. Using an N-terminal E2f3a-specific antibody, we detected E2f3a protein within NPCs in the ganglionic eminence (GE), a ventrally located tissue that gives rise to inhibitory interneurons (Wonders and Anderson, 2006), as well as the VZ and SVZ surrounding the lateral ventricle (Figures S4A and S4B). Importantly, E2f3a colocalizes with a subset of Sox2-expressing cells in the GE (Figure S4C) and the dorsal cortex (Figures 2E, 2G, and 2H) (also marked by Pax6 [Figure S4D]). Conversely, little E2f3a colocalization was found in committed BPs, which express Tbr2 (Figures 2F–2H), or in $Tuj1^+$ neurons (Figure 2H). Quantification of cells colabeled with E2f3a and cell-cycle phase markers revealed that E2f3a is highly enriched in S phase, during which 83% of $E2f3a^+$ cells coexpressed BrdU following a 2 hr pulse (Figures S4E–S4G). E2f3a expression in S phase precursors supports a role in NPC-fate decisions, given that a recent study suggests that fate decisions in the developing brain are controlled by gene expression patterns during S phase (Arai et al., 2011). Thus, E2f3a is expressed predominantly in $Sox2^+$ self-renewing precursors.

E2f3a Is Required for Regulation of NSC Self-Renewal

We asked next whether E2f3 isoforms modulate the self-renewal capacity of the stem cell-like AP population. Loss of E2f3a increased the number of primary and secondary neurospheres generated by both cortical and GE-derived NPC populations by 1.4- to 2-fold at E14.5 (Figure 2I) and E17.5 (Figure S4H). Loss of E2f3b, however, showed no effect (Figure 2J; Figure S4I). To ask whether E2f3a deficiency affects the mode of AP cell division, we measured the orientation of mitotic spindle poles in control and E2f3a-deficient brains. APs undergo mitosis at the apical surface of the lateral ventricle, and the orientation of the mitotic spindle pole and cleavage furrow during cytokinesis has been linked with the resulting fate of daughter cells (Das and Storey, 2012; Farkas and Huttner, 2008; Godin et al., 2010) (see Supplemental Information for detailed methods). In E2f3a knockouts, we observed 1.5-fold more APs with a cleavage angle within the vertical 75°–90° range, associated with symmetric (self-renewing) cell divisions (Figures S4J and S4K). In contrast, there was a corresponding 2.7-fold decrease in the number of divisions within the 0°–15° range, suggesting a reduction in asymmetric, differentiative cell divisions. These results suggest that E2f3a-deficient brains exhibit an increased proportion of AP cells undergoing symmetric cell divisions, consistent with our in vitro studies showing enhanced neural precursor self-renewal.

(E) Colocalization of E2f3a (green) with Sox2 (red) in the dorsal cortex (E14.5).
(F) Lack of colocalization between E2f3a (red) and the BP marker Tbr2 (green) in the dorsal cortex (E14.5).
(G) Quantification of the percentage of all $E2f3a^+$ cells per section in the dorsal cortex (E14.5) coexpressing Sox2, Pax6, Tbr2, or Tuj1 (n = 3).
(H) Quantification of the percentage of Pax6-, Sox2-, or Tbr2-expressing cells per section in the dorsal cortex (E14.5) that also express E2f3a (n = 3).
(I and J) Increased number of primary and secondary neurospheres in *E2f3a*$^{-/-}$ precursors derived from both GE and dorsal cortex (CTX) (I); E2f3b knockouts generate the same number of neurospheres as wild-types (J). Included in the right side are phase-contrast images of neurospheres from the indicated genotypes (n = 5–7).
For (A)–(J), results are presented as the mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The scale bar represents 100 μm. See also Figures S2–S24.

Opposing Regulation of the *Sox2* Gene by E2f3 Isoforms

To identify target genes through which E2f3 isoforms regulate NPC properties, we performed a genomic chromatin immunoprecipitation (ChIP)-on-chip screen to identify E2f3 binding sites and associated target genes in NPCs (L.M.J., Y. Liu, D.S.P., R.S.S., and A.B., unpublished data). From three independent samples of wild-type, *E2f3a*$^{-/-}$, and *E2f3b*$^{-/-}$ E14.5 GE neurospheres, we identified the gene encoding the pluripotency factor *Sox2* as a potential target of E2f3 (Figure 3A). Enrichment levels for E2f3 at the *Sox2* promoter were comparable in wild-type and E2f3a- and E2f3b-deficient cells, indicating that both isoforms bind this locus. Previous studies have shown that changes in Sox2 expression can have dramatic effects on the maintenance and differentiation capacity of neural precursor populations, wherein elevated Sox2 leads to expansion of the precursor pool and impaired neurogenesis, and decreased Sox2 results in loss of NPCs and dose-dependent defects on neurogenesis (Bani-Yaghoub et al., 2006; Graham et al., 2003; Pevny and Nicolis, 2010; Taranova et al., 2006). As precursor numbers and neurogenesis are disrupted in E2f3a- and E2f3b-deficient brains, *Sox2* was a strong candidate to account for these biological effects. We first validated by conventional ChIP that E2f3 binds to the *Sox2* promoter, at an enrichment level comparable to that of previously established E2f3 target genes (Figure 3B). An E2f consensus motif (CTTCCCGC) was identified within the center of the E2f3 binding peak, 371 bp upstream of the transcriptional start site (TSS) (Figure 3A), and is conserved in the murine and human genomes. This E2f3-bound region is transcriptionally responsive to E2f3 activity, as indicated by a 2-fold increase in luciferase activity from a *Sox2* promoter fragment (800 bp upstream to 285 bp downstream of the *Sox2* TSS) following cotransfection of a full-length E2f3 construct (Figures 3C and 3D). Furthermore, point mutations within the E2f consensus motif reduced luciferase activity by 50% (Figures 3C and 3D), demonstrating a functional E2f consensus site at 371 bp upstream of the *Sox2* TSS.

To determine whether E2f3 isoforms regulate Sox2 levels in vivo and in vitro, we measured Sox2 protein levels in GE-derived tissue and cultured neurospheres, because NPCs from this region are predominantly Sox2^{+} (Figures S5A and S5B). We show that E2f3a and E2f3b regulate Sox2 expression in a reciprocal manner. Specifically, *E2f3a*$^{-/-}$ neurospheres and GE tissue exhibited a 2.3- and 6-fold increase, respectively, in Sox2 levels (Figures 3E and 3F). In contrast, *E2f3b*$^{-/-}$ neurospheres and GE tissue express Sox2 at 40% and 30% of wild-type levels (Figures 3G and 3H). These results suggest an opposing role for E2f3 isoforms in the regulation of the *Sox2* gene.

E2f3a Represses NSC Self-Renewal through *Sox2* Regulation

To directly determine whether E2f3a represses self-renewal by regulating *Sox2*, we asked whether *Sox2* knockdown could rescue the enhanced self-renewal phenotype observed in E2f3a-deficient cultures. *E2f3a*$^{+/+}$ or *E2f3a*$^{-/-}$ neurospheres were infected with a bicistronic lentivirus expressing GFP and one of two short hairpin Sox2 (shSox2) or scrambled control sequences. Importantly, each shSox2 construct reduced Sox2 expression in *E2f3a*$^{-/-}$ cells to a level comparable to that of GFP-infected wild-type cells (Figures 4A and 4B). shSox2-mediated knockdown of *Sox2* in *E2f3a*$^{-/-}$ cultures restored neurosphere numbers (Figures 4C and 4D) and self-renewal capacity (Figure 4E) back to basal levels. To determine whether elevated Sox2 can account for the increased self-renewal in *E2f3a*$^{-/-}$ precursors, we overexpressed Sox2 in wild-type cultures (Figure 4F). Sox2 overexpression in *E2f3a*$^{+/+}$ precursors increased self-renewal (Figure 4G) and correspondingly decreased neurogenesis (Figure 4H) to levels observed in E2f3a-deficient cells. Furthermore, overexpression of Sox2 in *E2f3a*$^{-/-}$ precursors, which already express elevated Sox2, did not increase neurosphere numbers further. Thus, E2f3a functions to maintain Sox2 levels below a specific threshold, beyond which precursor self-renewal and cell-fate decisions are markedly disrupted.

E2f3 Isoforms Recruit Distinct Transcriptional Cofactors to the *Sox2* Promoter

To determine the mechanism by which E2f3a and E2f3b antagonistically regulate Sox2 expression, we identified the regulatory factors recruited to the *Sox2* locus by each isoform. We confirmed that both isoforms bind the *Sox2* promoter within a 200 bp region surrounding the conserved E2f motif (upstream binding site [US]) and at the TSS, given that E2f3 enrichment is similar in wild-type, *E2f3a*$^{-/-}$, and *E2f3b*$^{-/-}$ neural precursors (Figure 5A). Consistent with E2f3b as an activator of *Sox2* expression, in *E2f3a*$^{-/-}$ cells, in which only the E2f3b isoform is present, we observed enrichment of RNA polymerase II (Pol II) at and beyond the TSS (Figure 5B) and the trimethyl-H3K4 (H3K4me3) chromatin modification (Figure 5C), as well as a decrease in H3K27me3 (Figure 5C). Each of these changes are associated with transcriptional activation, demonstrating that in the absence of E2f3a, Sox2 expression is elevated due to an increased ratio of bound E2f3b-Pol II complexes. Conversely, binding of the repressive pocket protein p107 was significantly enriched in the absence of E2f3b, where only E2f3a is present, and was decreased in *E2f3a*$^{-/-}$ cells (Figure 5D). These findings show that E2f3a functions as a repressor at the *Sox2* promoter by recruiting p107. Supporting this conclusion, GE tissue from p107-deficient animals exhibited a 2.2-fold increase in Sox2 levels compared to wild-type controls (Figure 5E).

The percentage of precursor cells in each cell-cycle phase was not altered by E2f3a or E2f3b deficiency (Figures S6A and S6B); thus, the changes in binding enrichments we observed in E2f3 isoform-deficient cells could not be explained by disrupted cell-cycle dynamics, but rather by altered enrichment of these factors. We show that E2f3 isoforms regulate *Sox2* expression in an opposing manner, whereby E2f3a recruits the transcriptional repressor p107 and E2f3b recruits activator complexes to the promoter. This reveals a novel mechanism for regulation of the pluripotency factor *Sox2*, through the cell cycle effectors E2f3a and E2F3b.

A Common Mechanism of E2f3a-Mediated *Sox2* Regulation in Embryonic and Adult NSCs

The requirement for controlled Sox2 expression in NSCs extends from development to adulthood (Cavallaro et al., 2008; Favaro et al., 2009; Ferri et al., 2004; Pevny and Nicolis,

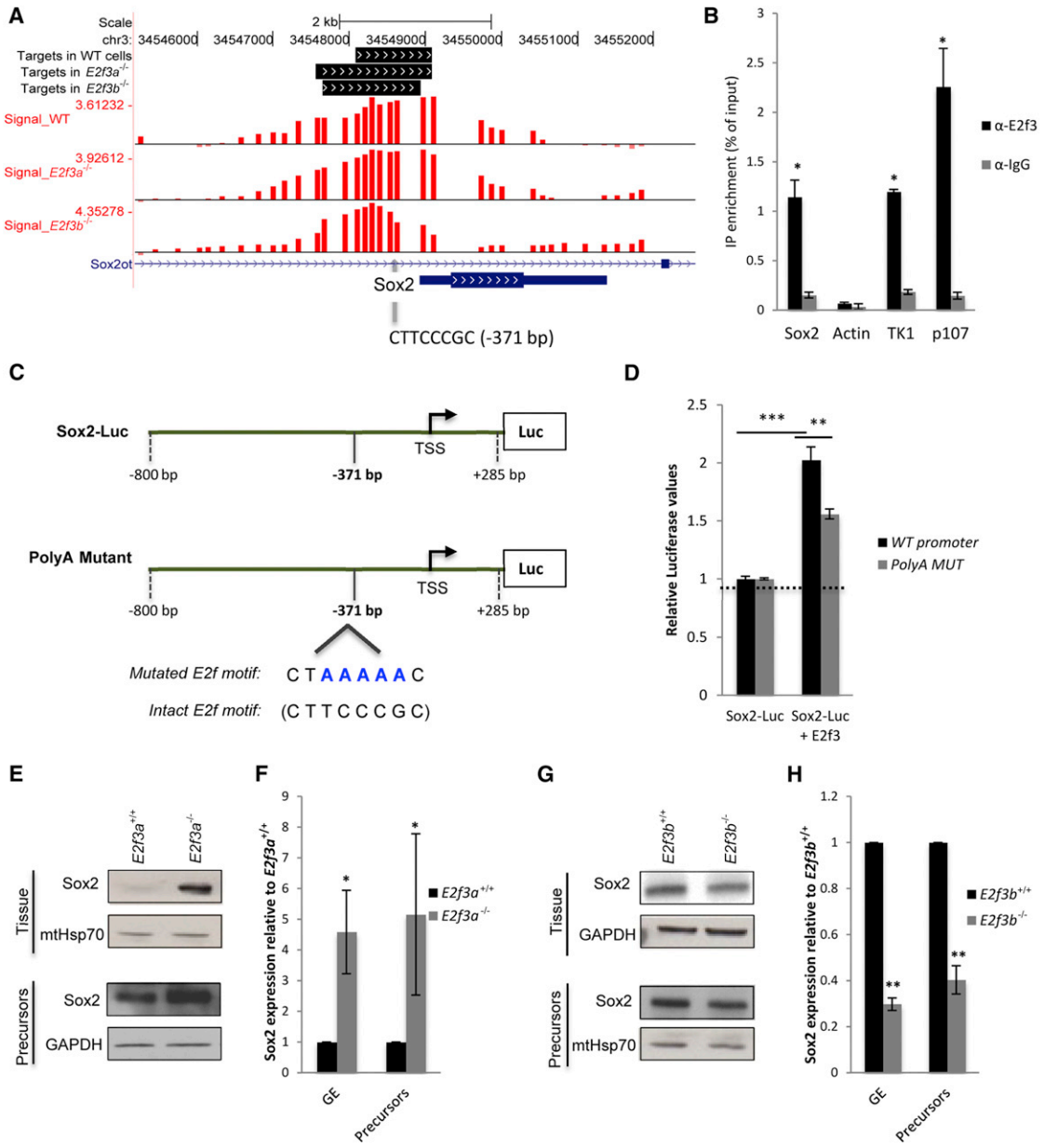

Figure 3. E2f3 Isoforms Regulate *Sox2* Expression in an Opposing Manner

(A) Binding-peak profiles for E2f3 from *E2f3a*$^{+/+}$, *E2f3a*$^{-/-}$, and *E2f3b*$^{-/-}$ ChIP-on-chip experiments, generated with the UCSC Genome Browser (http://genome.ucsc.edu/). E2f3 binding peaks extend throughout the proximal promoter region and the TSS. An E2f consensus motif was identified at 371 bp upstream of the TSS. WT, wild-type.

(B) RT-PCR analysis of E2f3 ChIP experiments shows E2f3 binding at the *Sox2* promoter with an enrichment value similar to that for other known E2f targets. Plotted is the mean from at least three independent experiments ± SEM (n = 4).

(C) Model for luciferase experiments. E2f3-dependent activity was tested from a Sox2 promoter fragment covering −800 bp to +285 bp relative to the TSS. For E2f consensus site mutation, five core nucleotides were replaced with adenine.

(D) E2f3a drives Sox2-luciferase activity. Mutation of the E2f consensus motif reduced E2f3-mediated transcription by 50% (n = 4–6).

(E and G) Immunoblot for Sox2 from cultured E2f3a (E) or E2f3b (G) neural precursors or GE tissue. GAPDH and mtHsp70 were included as protein-loading controls.

(F and H) Quantification by densitometry of immunoblots shows that E2f3a knockouts express significantly more Sox2 (F), whereas E2f3b knockouts have lower Sox2 levels (H).

For all quantifications, data are plotted as the mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001). See also Figure S5.

2010); thus, we hypothesized that E2f3-dependent *Sox2* regulation is also important in adult NSCs. To evaluate the role of E2f3a in the adult we generated animals containing two modified E2f3 alleles: one *floxed* allele and a second E2f3a-deficient allele (*E2f3-flox/3a*$^{-}$). To acutely remove E2f3a, we infected cultured SVZ precursors from adult *E2f3-flox/3a*$^{-}$ animals with

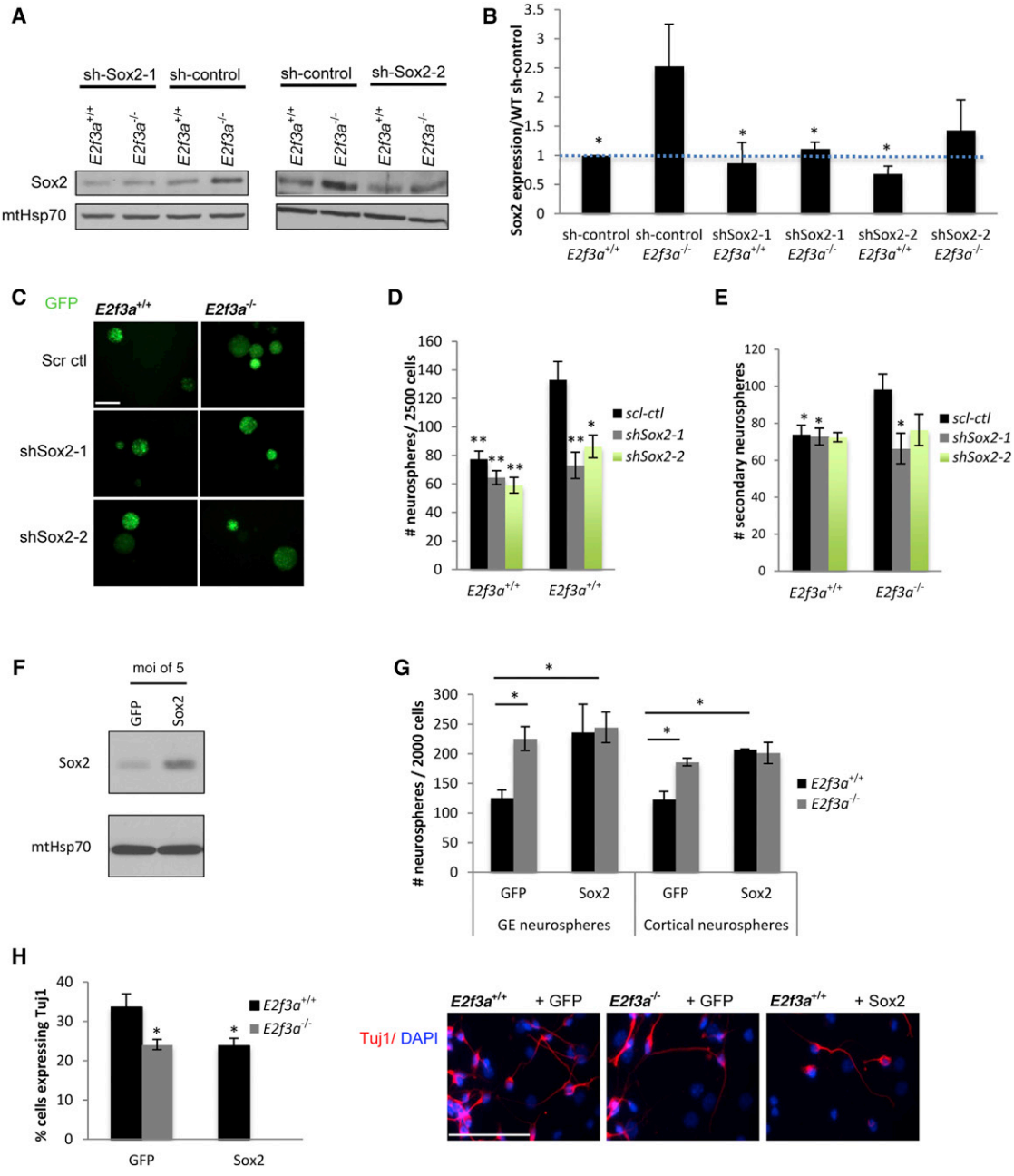

Figure 4. Regulation of NSC Self-Renewal by E2f3a Is Sox2-Dependent

(A and B) Immunoblot analysis (A) and densitometry quantification (B) of Sox2 expression in cultured GE neurospheres (E14.5) 5 days post infection (p.i.) with scrambled (Scr) control or shSox2 lentiviruses (n = 3). mtHsp70 was included as a measure of protein loading.

(C) Representative images of GFP-positive neurospheres 7 days p.i. Scale bar represents 200 μm.

(D and E) Infected cultures were plated immediately for primary neurosphere assays (D) and one week later were used in secondary neurosphere assays (E). Neurosphere numbers are restored in E2f3a knockouts following *Sox2* knockdown (n = 4).

(F) Immunoblot demonstrating increased Sox2 expression in neurospheres infected with a Sox2-expressing lentivirus compared to GFP-infected cells, 4 days p.i.

(G) Cells were plated 7 days p.i. for secondary neurosphere assays. Sox2 overexpression in wild-type cells increases self-renewal (n = 3).

(H) *E2f3a*$^{+/+}$ and *E2f3a*$^{-/-}$ neurospheres were cultured in differentiation media on poly-L-ornithine plates 7 days p.i. and were fixed and stained for Tuj1 and DAPI after 6 days. The percentage of DAPI^{+} cells that express Tuj1 was quantified. Sox2 overexpression inhibits neuronal differentiation in vitro. Scale bar represents 50 μm (n = 3).

Significance was determined for all samples compared to *E2f3a*$^{-/-}$ (B, D, and E) or *E2f3a*$^{+/+}$ (G and H) cells infected with control virus. All data are presented as the mean ± SEM (*p < 0.05, **p < 0.01).

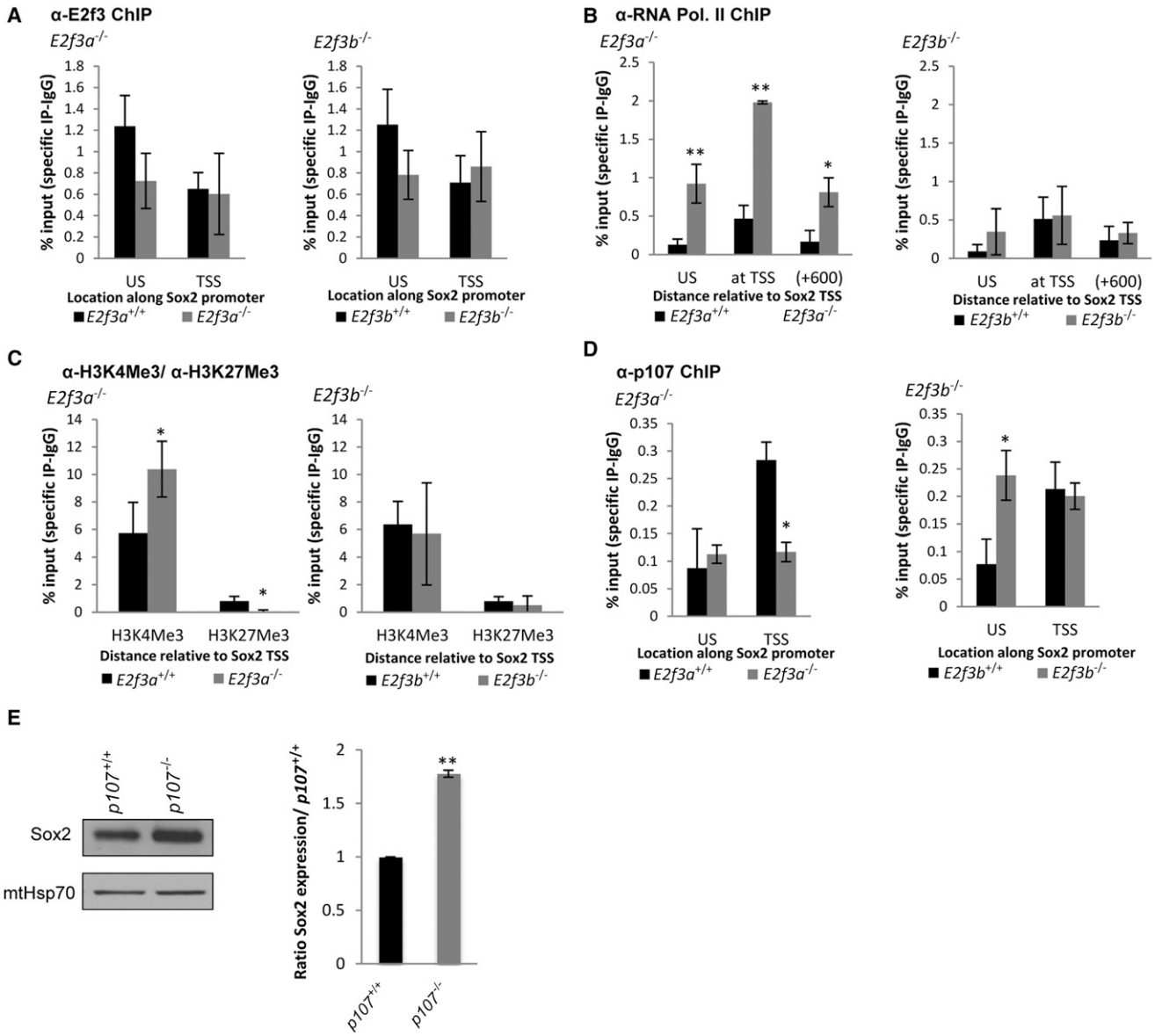

Figure 5. E2f3 Isoforms Recruit Distinct Transcriptional Cofactors to the *Sox2* Promoter

(A–D) ChIP was performed in *E2f3a*$^{-/-}$, *E2f3b*$^{-/-}$, and wild-type (both *E2f3a*$^{+/+}$ and *E2f3b*$^{+/+}$) GE-derived neurospheres using antibodies for E2f3 (A), Pol II (B), H3K4me3 and H3K27me3 (C), and p107 (D). Chromatin enrichment was quantified by RT-PCR using primers designed to amplify 200 bp regions centered on either the upstream (US) E2f binding motif or the TSS of the *Sox2* promoter. For all panels, we have plotted values for the specific antibody IP with immunoglobulin G (IgG) values subtracted (n = 3–5).

(E) Immunoblot analysis and densitometry quantification of *p107*$^{+/+}$ and *p107*$^{-/-}$ GE tissue shows a significant increase in Sox2 levels in the absence of p107 (n = 3). See also Figure S6.

Data for (A)–(E) are plotted as the mean ± SEM (*p < 0.05, **p < 0.01).

a Cre-expressing lentivirus, which removes *E2f3a* but leaves *E2f3b* intact (Figure 6A). As with embryonic *E2f3a*$^{-/-}$ precursors, Cre-infected cells exhibited increased neurosphere self-renewal (Figure 6B) and were impaired in their ability to generate neurons (Figure 6C). Importantly, these self-renewal and neurogenic changes were accompanied by increased Sox2 expression (Figure 6D). Furthermore, the absence of E2f3a reduced enrichment of E2f3 (Figure 6E) and p107 (Figure 6F) and significantly increased recruitment of Pol II (Figure 6G) at the *Sox2* promoter. Thus, NSCs maintain a common mechanism of E2f3a-dependent *Sox2* regulation from development to adulthood.

Loss of E2f3a Disrupts Neurogenesis and Cognitive Function in the Adult Brain

Given that E2f3a regulates *Sox2* in both embryonic and adult NSCs, we asked whether absence of E2f3a had a functional consequence in the adult brain. We evaluated the levels of

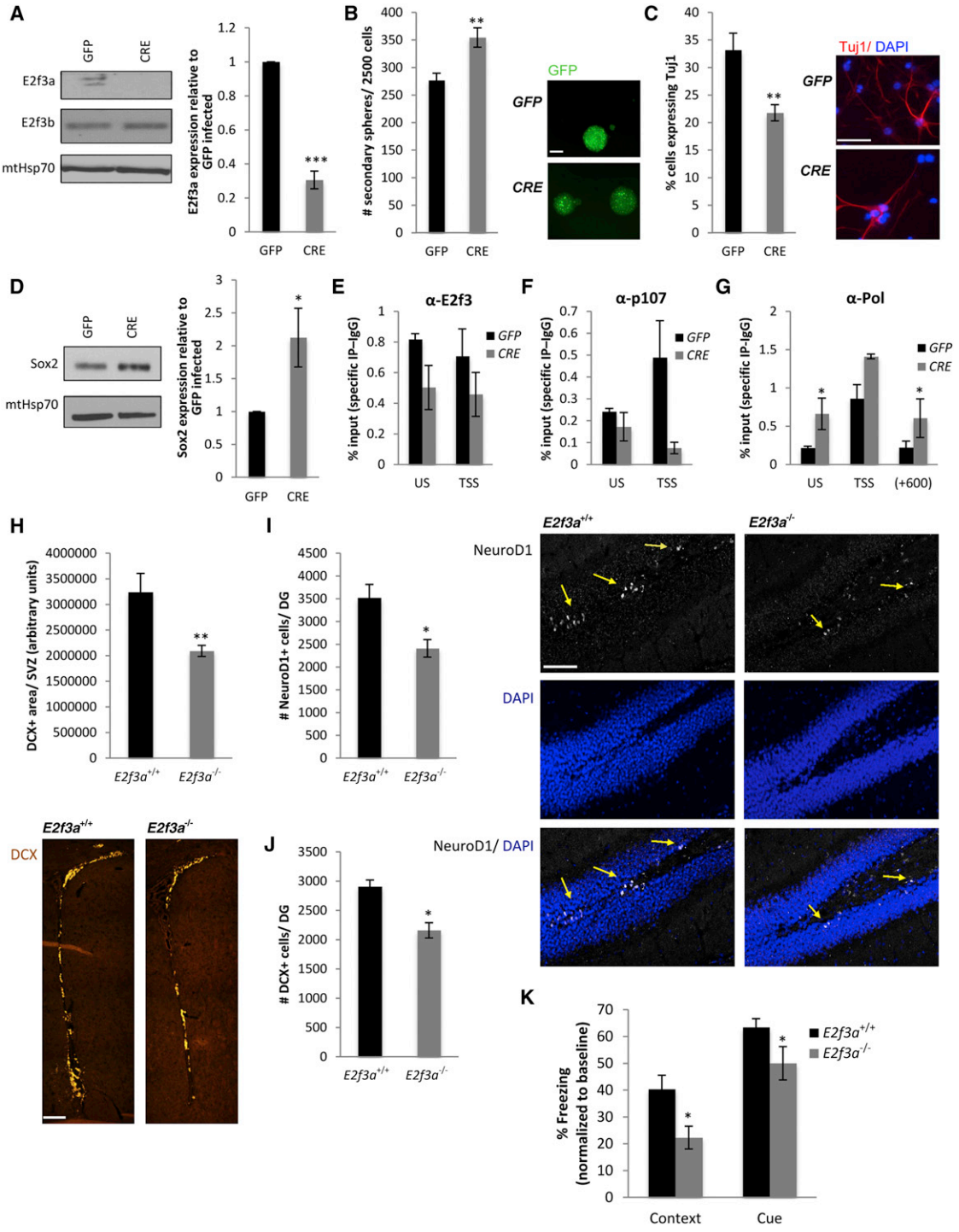

Figure 6. E2f3a Regulates *Sox2*, Neurogenesis, and Cognitive Function in the Adult Brain

(A) Immunoblot showing loss of E2f3a, but not E2f3b, in *E2f3-flox/E2f3a*$^{-}$ adult SVZ precursors 4 days after Cre infection. Results were quantified by densitometry, using mtHsp70 as a loading control (n = 4).

(B) Infected cells were plated for neurosphere assays 7 days p.i., and regenerated neurospheres were counted 6 days later. Cre-infected cells generated more neurospheres than did controls. The image to the right shows GFP-expressing neurospheres infected with either control (GFP) or GFP-CRE virus 7 days p.i. (n = 7).

(C) Neurospheres were plated in differentiation media on poly-L-ornithine-coated dishes 7 days p.i. and were fixed and stained for Tuj1 and DAPI after 6 days. The percentage of DAPI^{+} cells expressing Tuj1 was quantified. Cre-infected cells have a reduced capacity to generate neurons (n = 5).

(D) Elevated Sox2 in Cre- versus GFP-infected *E2f3-flox/E2f3a*$^{-}$ adult precursors (5 days p.i.), quantified by densitometry using mtHsp70 as a loading control (n = 3).

(E–G) RT-PCR analysis of ChIP assays for E2f3 (E), p107 (F), and Pol II (G) in GFP- or Cre-infected *E2f3-flox/E2f3a*$^{-/-}$ precursors (n = 4).

(legend continued on next page)

neurogenesis in *E2f3a*$^{+/+}$ and *E2f3a*$^{-/-}$ adult brains in two distinct neurogenic regions, the SVZ and the dentate gyrus (DG) of the hippocampus, wherein neurogenesis is required for olfactory function and hippocampal memory formation, respectively. As determined by NeuroD1 and DCX staining, the number of committed neurons was significantly decreased, by 35% in the SVZ (Figure 6H) and 31% in the DG (Figures 6I and 6J), revealing an impairment in adult neurogenesis that had progressed since the late stages of development. We also found that *E2f3a*$^{-/-}$ mice are significantly impaired in their ability to learn and remember the association between an aversive experience and environment in the classical fear-conditioning paradigm (Wehner and Radcliffe, 2004). In this test, animals are trained to acquire a learned response to an aversive stimulus (foot shock) that is associated with a specific environment (context) and a tone (cue). Following training, animals are tested for their ability to have learned that the context or cue is associated with the aversive stimulus through measuring their freezing behavior during exposure to each condition. *E2f3a*$^{-/-}$ mice exhibited a significant 45% decrease in freezing behavior associated with amygdala- and hippocampus-dependent contextual learning (Marín-Burgin and Schinder, 2012) and a more subtle 21% decrease in freezing associated with amygdala-dependent cue learning (Figure 6K) (Wehner and Radcliffe, 2004). The reduced freezing in *E2f3a*$^{-/-}$ versus control mice was not due to differences in the unconditioned freezing behavior, as assessed during training and before tone presentation in the cue trial (Figures S7A and S7B), nor to differences in the foot-shock threshold (Figure S7C). These results suggest that E2f3a influences the formation of associative memories and that its loss reveals defects in at least two telencephalic structures, with the most severely affected function (contextual learning) being that which is influenced by adult neurogenesis (Marín-Burgin and Schinder, 2012). Thus, E2f3a is required for regulation of neural precursor maintenance and neurogenesis in both the embryonic and adult brain, and this role significantly impacts cognitive function.

DISCUSSION

This study presents two key discoveries. First, we show that E2f3 isoforms play opposing roles in regulating the balance between neural precursor self-renewal and differentiation during developmental neurogenesis. Loss of E2f3a leads to neurogenic defects in adulthood, underscoring the importance of E2f3 in mediating fate choices in both embryonic and adult NSCs. Second, we report a transcriptional mechanism by which E2f3 isoforms antagonistically regulate levels of *Sox2* expression. Alteration of *Sox2* expression by loss of either E2f3 isoform shifts the equilibrium between precursor expansion and differentiation, thereby affecting downstream generation of cortical neurons and ultimately cognitive function.

Based on our findings and previous reports of E2f3 isoform expression patterns (Adams et al., 2000), we predict that E2f3a is predominant during S phase, whereas E2f3b is expressed throughout the cell cycle. Thus, at different phases of the cell cycle, E2f3a and E2f3b isoforms dynamically fine-tune Sox2 expression levels. We present a model of E2f3-dependent *Sox2* regulation in which both E2f3 isoforms, in a see-saw-like fashion, regulate levels of Sox2 in proliferating NSCs to ensure the proper balance of precursor expansion and differentiation (Figure 7A). When E2f3b is lost, E2f3a-p107-mediated repression is not balanced by E2f3b-mediated activation, leading to lower Sox2 levels and increased neurogenesis at the expense of precursor expansion (Figure 7B). Conversely, E2f3a deficiency leads to activation by E2f3b that is not balanced by E2f3a-mediated repression, resulting in elevated Sox2 levels and, consequently, increased precursor self-renewal at the expense of neurogenesis (Figure 7C). This functional model illustrates the requirement for a balance between E2f3a and E2f3b transcriptional activities to maintain the correct dosage of Sox2 in stem and progenitor cells.

Although E2f3a, E2f3b, and p107 are highly expressed in NPCs, these proteins become rapidly downregulated as cells undergo differentiation. Uncovering other mechanisms by which long-term repression of *Sox2* is maintained as cells differentiate will therefore be important. Notably, a recent study has shown that the Cyclin-dependent kinase inhibitor (CKI) p27 is required for repression of *Sox2* during the differentiation of pluripotent stem cells (Li et al., 2012). p27 is recruited to the *Sox2* SRR2 enhancer and functions in a complex together with p130 and E2f4 to silence *Sox2* expression during differentiation. As the pocket protein p130 is highly expressed in differentiated cells and plays a key role in long-term silencing of cell-cycle genes, it may well play a crucial role in silencing *Sox2* in postmitotic neurons. Another recent study demonstrated that the CKI p21 represses *Sox2* expression in adult NSCs (Marqués-Torrejón et al., 2013). In adult NSCs it has been shown that p21 represses *Sox2* through the SRR2 enhancer and that its loss results in excessive *Sox2* expression and precursor exhaustion. This exhaustion, however, is preceded by an initial expansion of the precursor pool (Kippin et al., 2005; Marqués-Torrejón et al., 2013). In E2f3a-deficient embryonic and adult NSCs, we found that elevated levels of Sox2 overexpression lead to increased self-renewal; however, it is also possible that E2f3a-deficient NSCs may exhaust with time, following extended passaging in vitro or with advanced animal age. It is also conceivable that E2f3 may participate in the recruitment of p21 to its SRR2-enhancer binding site. However, given that the E2f3 and p21 binding sites have been identified in distinct regulatory domains of the *Sox2* gene and that E2f-independent mechanisms of p21 recruitment to *Sox2* have been suggested (Marqués-Torrejón et al., 2013), it is probable that they regulate *Sox2* expression by distinct mechanisms. Examining these questions will be an

(H–J) Neurogenesis was measured in the adult brain by quantifying the total area of DCX staining along the SVZ (n = 4) (H) and the number of NeuroD1$^+$ or DCX$^+$ cells in the DG of the hippocampus (n = 3) (I and J).
(K) *E2f3a*$^{-/-}$ mice spent 45% and 21% less time freezing in the context ($p = 0.015$) and after the auditory cue ($p = 0.047$), respectively, following fear-conditioning training (n = 18 for *E2f3a*$^{+/+}$; n = 13 for *E2f3a*$^{-/-}$).
For (A)–(K), data are presented as the mean ± SEM. Scale bar represents 100 μm for (B), (H), and (J) and 25 μm for (C) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). See also Figure S7.

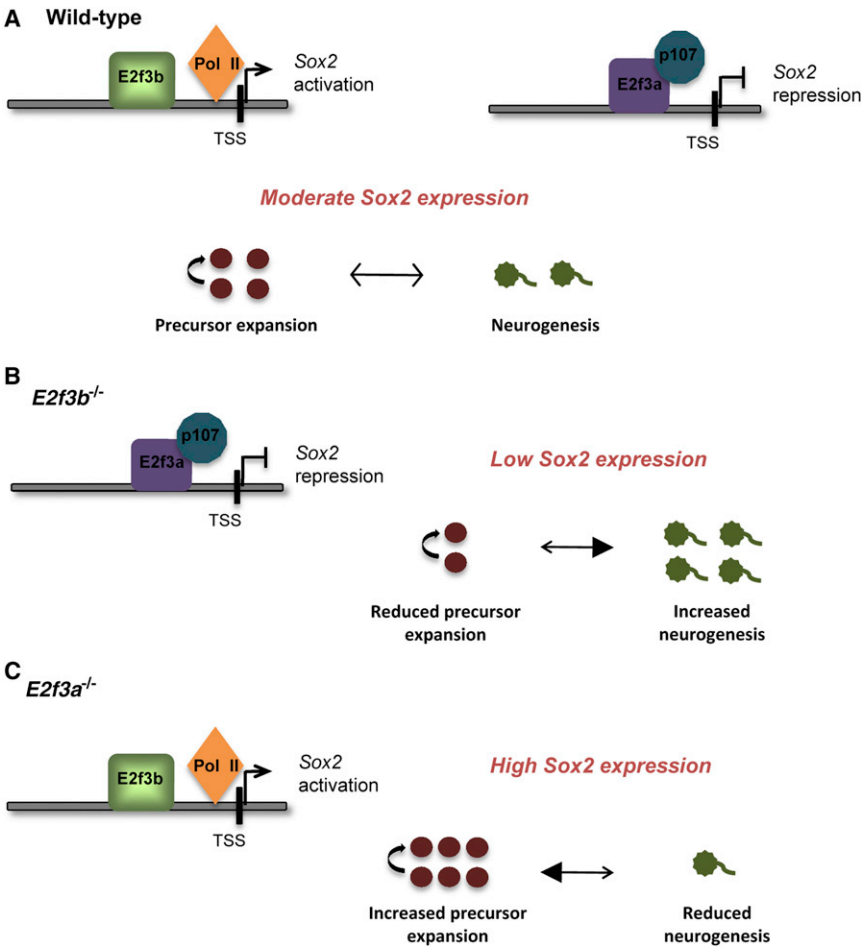

Figure 7. Model of E2f3 Function: E2f3 Isoforms Regulate *Sox2* Transcription in an Opposing Manner to Direct NSC Fate Choice

(A) In wild-type conditions, E2f3b activates and E2f3a represses *Sox2* transcription, allowing for dynamic fine-tuning of Sox2 levels. This fine-tuning maintains the proper balance between neurogenesis and expansion of the NPC pool.

(B) In the absence of E2f3b, E2f3a-p107-mediated repression dominates and reduces Sox2 levels, thereby increasing neurogenesis at the expense of precursor expansion.

(C) In the absence of E2f3a, E2f3a repression unbalances E2f3b-mediated activation, thus increasing Sox2 levels and promoting NPC expansion at the expense of neurogenesis.

important focus for future studies and will contribute to our understanding of molecular events underlying *Sox2* gene silencing in differentiating cells.

Through our identification of *Sox2* as a functional target gene of the pRb/E2f pathway in neural precursors, we have linked the cell-cycle machinery with pluripotent gene regulation in a biologically relevant context. E2f-dependent regulation of *Sox2* has clear functional consequences in the developing brain; however, we suggest that this may be a common feature of stem cell regulation and the pluripotent state, given that both Sox2, pRb, and E2f proteins are expressed in diverse tissue-specific, pluripotent, and cancer stem cell populations (Arnold et al., 2011; Galderisi et al., 2006; Pevny and Nicolis, 2010). In addition, the pRb binding proteins RBBP4 and RBBP9 have recently been implicated in regulation of the pluripotent state in human stem cells, and E2f motifs have been identified in the promoters of key pluripotency factors, including *NANOG*, *POU5F1*, *FOXD3*, and *SOX2* (O'Connor et al., 2011). ChIP-based experiments have further demonstrated that E2fs are found at the promoters of a large number of pluripotency-related genes (Chen et al., 2008; O'Connor et al., 2011), although direct functional consequences for these interactions have not previously been described. In conclusion, these studies point to the possibility that E2fs may regulate other pluripotency factors in addition to *Sox2* and support the idea that E2f3-dependent *Sox2* regulation is a fundamental mechanism in tissue-specific, tumorigenic, and pluripotent stem cell populations.

EXPERIMENTAL PROCEDURES

Mouse Models

Germline *E2f3a*- and *E2f3b*-null mice were originally generated by G. Leone and were maintained on an FVB/N background (Chen et al., 2007; Tsai et al., 2008). Animal experiments were approved by the University of Ottawa's Animal Care Committee, which abides by the guidelines of the Canadian Council on Animal Care. *E2f3-flox/E2f3a*$^{-}$ mice were generated by crossing *E2f3a*$^{-/-}$ mice with *E2f3-flox/flox* animals maintained on an FVB/N background. p107-deficient mice were generated as previously described (LeCouter et al., 1998). All adult mice analyzed were 2 months of age or older.

Neural Precursor Cultures

Neural precursors were obtained by dissection of the ventral (GE) or dorsal (cortex) telencephalic tissue of developing embryos; neurosphere and in vitro differentiation assays were performed as previously described (Vanderluit et al., 2004), with the exception of the lentivirus experiments, in which neural precursors were plated at a density of 5 cells/μl 7 days post infection and the number of regenerated neurospheres were counted after 6 days in culture. All neurosphere assays were performed with brain samples from four to seven independent animals, from at least two separate experiments.

Western Blotting, Immunohistochemistry, Cell Counts, Primers, and Antibodies

Details are described in Supplemental Experimental Procedures.

Statistical Analysis

All statistical comparisons in this study were performed using an unpaired two-tailed t test. Differences were considered significant with a p value of <0.05 (*), **p < 0.01, ***p < 0.001.

Lentiviral Infections

shRNA lentiviral expression constructs were obtained from Open Biosystems and included scrambled control (catalog no. RHS4346), shSox2-1 (clone ID 153337), and shSox2-2 (clone ID 153339) plasmids. Neurosphere cultures were infected with lentiviral particles at a multiplicity of infection (moi) of 30. For Sox2 overexpression, the *Sox2* coding sequence was subcloned into the multiple cloning site (MCS) of an internal ribosome entry site-GFP

backbone, viruses were produced, and neurospheres were infected at an moi of 5. For Cre-expression experiments, GFP- or Cre-coding sequences were subcloned into the MCS of a pWPXLD plasmid, and cells were infected at an moi of 10.

Luciferase Reporter Assays

Reporter assays were performed in HEK293T cells as previously described (Andrusiak et al., 2011). E2f consensus site mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit and primer-design software from Stratagene.

ChIP

ChIP analysis was performed as previously described (Andrusiak et al., 2011) in proliferating neurospheres, except that immunocomplexes were captured using Dynabeads Protein A. RT-PCR was used to quantify ChIP enrichment values. Each experiment was performed on at least three independent samples. ChIP-on-chip experiments were performed as previously described (Liu et al., 2010).

Fear-Conditioning Analysis

Details are described in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.02.001.

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APPENDIX E

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Co-author Publications – first page

Development/Plasticity/Repair

The Rb/E2F Pathway Modulates Neurogenesis through Direct Regulation of the *Dlx1/Dlx2* Bigene Cluster

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During brain morphogenesis, the mechanisms through which the cell cycle machinery integrates with differentiation signals remain elusive. Here we show that the Rb/E2F pathway regulates key aspects of differentiation and migration through direct control of the Dlx1 and Dlx2 homeodomain proteins, required for interneuron specification. Rb deficiency results in a dramatic reduction of *Dlx1* and *Dlx2* gene expression manifested by loss of interneuron subtypes and severe migration defects in the mouse brain. The Rb/E2F pathway modulates *Dlx1/Dlx2* regulation through direct interaction with a *Dlx* forebrain-specific enhancer, I12b, and the *Dlx1/Dlx2* proximal promoter regions, through repressor E2F sites both *in vitro* and *in vivo*. In the absence of Rb, we demonstrate that repressor E2Fs inhibit *Dlx* transcription at the *Dlx1/Dlx2* promoters and *Dlx1/2*-I12b enhancer to suppress differentiation. Our findings support a model whereby the cell cycle machinery not only controls cell division but also modulates neuronal differentiation and migration through direct regulation of the *Dlx1/Dlx2* bigene cluster during embryonic development.

Introduction

During brain development, cell cycle regulation and differentiation are tightly coordinated developmental programs. Cross talk exists between the cell cycle machinery and differentiation pathways to ensure that progenitor populations are maintained and that differentiation is induced at the time of terminal mitosis (McConnell and Kaznowski, 1991; Nguyen et al., 2006; Farkas and Huttner, 2008; Frank and Tsai, 2009). Despite the importance of the precise coordination of these events, the mechanisms by which the cell cycle machinery integrates with differentiation signals remain poorly understood.

The retinoblastoma protein, pRb, is a tumor suppressor gene that controls the G_1-S phase checkpoint during cell cycle regulation (McClellan and Slack, 2006; Chen et al., 2009; Freedman et al., 2009). Rb regulates the transcription of genes that are required for DNA replication and cell cycle progression by binding and inhibiting E2F transcription factors (Burkhart and Sage, 2008). There are eight E2Fs, five of which can bind Rb (E2F1–5) and are considered among the classical Rb partners while E2F6–8 are Rb-independent repressors (Dick and Dyson, 2006; Chen et al., 2009; Lammens et al., 2009). E2F1, 2, and 3 are primarily transcriptional activators while E2F4 and 5 repress transcription and induce gene silencing through pocket protein binding (Dick and Dyson, 2006). E2F7 and E2F8, two atypical E2Fs (Lammens et al., 2009), can form homo and heterodimers which, in the absence of pocket proteins, bind and repress E2F target genes. The expression of E2F7 and 8 is induced by activating E2Fs and are believed to serve as a fine tuning mechanism to modulate E2F target gene regulation (Di Stefano et al., 2003; Christensen et al., 2005; Lammens et al., 2009).

It has been proposed that the Rb pathway may have novel function(s) that extend beyond cell cycle control (McClellan and Slack, 2006, 2007). Conditional knock-out studies have suggested that Rb may have a role in regulating differentiation and migration (Takahashi et al., 2003; Ferguson et al., 2005; Chen et al., 2007; McClellan et al., 2007); however, the underlying mechanisms remain unknown. Clearly, indirect cross talk between the cell cycle machinery and differentiation pathways is essential to prevent premature differentiation of proliferating progenitors while promoting differentiation as cell division ceases. If the cell cycle proteins themselves could directly regulate genes required for differentiation, these two processes could become intimately coordinated.

Here we have uncovered a more direct role for cell cycle proteins in neuronal differentiation through the control of Dlx1 and Dlx2 homeodomain protein regulation, two key proteins that specify GABAergic neurons in the brain. Consistent with a deficit in *Dlx1/Dlx2* gene expression, mice lacking Rb exhibited a pro-

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Rb/E2F Regulates Expression of Neogenin during Neuronal Migration▽

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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

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The p107/E2F Pathway Regulates Fibroblast Growth Factor 2 Responsiveness in Neural Precursor Cells▽

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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

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